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Role of mitochondrial dysfunction in the development of nutrient-induced hyperinsulinemia

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ROLE OF MITOCHONDRIAL DYSFUNCTION IN THE DEVELOPMENT OF NUTRIENT-INDUCED HYPERINSULINEMIA

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DEDICATION

This work is dedicated to my parents. I am blessed with their unconditional love, encouragement, and support.

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ROLE OF MITOCHONDRIAL DYSFUNCTION IN DEVELOPMENT OF NUTRIENT-INDUCED HYPERINSULINEMIA

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ABSTRACT

Pancreatic beta cells sense fluctuations in circulating nutrients and adjust the rate of insulin secretion to maintain glucose homeostasis. Mitochondria integrate changes in nutrient flux to the generation of signals that modulate insulin secretion via oxidative phosphorylation. Type 2 Diabetes (T2D) is characterized by beta cell mitochondrial dysfunction and impairment of insulin secretion. Early stage progression of this disease in obese and pre-diabetic subjects is characterized by basal hypersecretion of insulin and increased insulin resistance in peripheral tissues including muscle, liver and adipose tissue. Whether basal hypersecretion of insulin or insulin resistance is the primary defect in T2D progression is still debated. The molecular mechanism underlying basal insulin hypersecretion and how it may lead to beta cell failure are not understood. Herein, we optimize a model of glucolipotoxicity that results in increased basal and reduced stimulated insulin secretion response. Furthermore, we show that pancreatic islets exposed to excess nutrients *in vitro* or isolated from high fat diet fed animals, have a decreased bioenergetic efficiency, which is characterized by

increased mitochondrial proton leak. Leak represents the fraction of oxygen consumed that is not coupled to ATP production. We show that leak is sufficient to induce insulin secretion at basal glucose levels and that nutrient-induced insulin secretion at basal glucose is leak-dependent. Finally, we identify the mitochondrial permeability transition pore (PTP) as the source of the leak. Our findings suggest the PTP may be a potential therapeutic target to prevent/delay the onset of hyperinsulinemia in pre-diabetic subjects.

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LIST OF ABBREVIATIONS

ADP ..... Adenosine diphosphate

ANT ..... Adenine nucleotide transporter

ATP..... Adenosine triphosphate

CPT1 ..... Carnitine Palmitoyl Transferase I

CsA..... Cyclosporin A

CypD..... Cyclophilin D

DAG..... Diacylglycerol

DNP ..... Dinitrophenol

DZ..... Diazoxide

ER..... Endoplasmic Reticulum

ETC ..... Electron Transport Chain

FA ..... Fatty Acid

FAO ..... Fatty Acid Oxidation

FBG ..... Fasting blood glucose

FBS..... Fetal bovine serum

FCCP..... Carbonyl Cyanide P-trifluoromethoxyphenylhydrazone

FFA..... Free Fatty Acid

G3P ..... Glycerol 3 Phosphate

G6P ..... Glucose 6-phosphate

GK ..... Glucokinase

GL..... Glycerolipid

GLT ..... Glucolipotoxicity

GLUT ..... Glucose transporter

GPR40 ..... G-protein coupled receptor 40

GSIS ..... Glucose-Stimulated Insulin Secretion

HI ..... Hyperinsulinemia

IGT ..... Impaired glucose tolerance

IR ..... Insulin Resistance

K_{ATP} ..... ATP-sensitive K^+ channel

KCl ..... Potassium chloride

KREBS ..... Krebs-ringer Bicarbonate Buffer

LC ..... Long-Chain

MAG ..... Monoacylglycerol

mGTP ..... Mitochondrial guanosine triphosphate

mtDNA ..... mitochondrial DNA

NN414 ..... Tifenazoxide

NNT ..... Nicotinamide nucleotide transhydrogenase

OCR ..... Oxygen consumption rate

OP ..... Oleate/Palmitate

OMM ..... Outer mitochondrial membrane

OCSP ..... Oligomycin sensitivity conferral protein

PDH ..... Pyruvate dehydrogenase

PEP ..... Phosphoenolpyruvate

PEPCK ..... Phosphoenolpyruvate carboxykinase

PK..... Pyruvate Kinase

PFK..... Phosphofructokinase

PPIF..... Peptidyl-prolyl cis-trans isomerase

PTP..... Permeability transition pore

ROS..... Reactive Oxygen Species

SNARE .. Soluble N-ethylmaleimide-sensitive Factor Attachment Protein Receptor

SUR1 ..... Sulfonyl urea receptor 1

T2D..... Type 2 Diabetes

TAG ..... Triacylglycerol

TCA ..... Tricarboxylic acid

TG..... Triglyceride

UCP ..... Uncoupling protein

VDAC..... Voltage-Dependent Ca^{2+} Channel

ZDF..... Zucker diabetic fatty

CHAPTER ONE: General Introduction

Diabetes Pathology, Prevalence

Type 2 Diabetes (T2D) is a chronic metabolic disease characterized by hyperglycemia (Golay & Ybarra, 2005). T2D increases the risk of cardiovascular disease and stroke and leads to neuropathy, retinopathy and kidney failure (Khalil, 2016; Sheetz & King, 2002). The rise in T2D prevalence both in the US and globally is increasing at an alarming rate (Guariguata et al., 2014; Roglic, 2016). The most recent International Diabetes Federation (IDF) data reports the global prevalence of diabetes at 8.8% in 2015 and is expected to rise to 10.4% by 2040 (Ogurtsova et al., 2017). The escalation in the incidence of diabetes creates a substantial economic burden due to increases in diabetes related medical care costs (Caspersen, Thomas, Boseman, Beckles, & Albright, 2012; Ogurtsova et al., 2017). This, in conjunction with a sharp rise in the prevalence of obesity, has created a need for extensive research to address the mechanisms involved in the progression of T2D. Diabetes is associated with development of insulin resistance and beta failure (Kasuga, 2006). The vast majority of research was focused on insulin resistance since this was thought to be the driver of diabetes. It has recently been shown, however, that the progression from pre-diabetes to diabetes may result from beta cell dysfunction (Page & Johnson, 2018). A clear understanding of the basic physiology of the pancreatic beta cell is

essential to elucidate the pathophysiology involved in diabetes development, leading to better therapeutics to prevent or delay progression of this epidemic disease.

Pancreatic β-cell Physiology

The pancreas is a critical organ that plays an important role in the digestive and endocrine systems. It has an endocrine and exocrine component. The exocrine pancreas produces digestive juices that flow from the pancreatic duct to the duodenum of the small intestine to enable digestion of food. The endocrine pancreas is composed of the Islets of Langerhans, which include β-cells, α-cells, δ-cells, and γ-cells. These cells secrete insulin, glucagon, somatostatin, and pancreatic polypeptide, respectively (Dolenšek, Rupnik, & Stožer, 2015). The secretion of glucagon by the α-cells and insulin by the β-cells is fundamental to proper blood glucose regulation, as they work inversely to each other to either increase or decrease blood glucose (Röder, Wu, Liu, & Han, 2016). Under fasting conditions, pancreatic α-cells stimulate the secretion of glucagon, promoting the breakdown of glycogen to glucose in the liver, which raises blood sugar back to normal. Conversely, pancreatic β-cells secrete insulin after ingestion of a meal to increase glucose uptake from the blood and into the peripheral tissues, stimulating glycogen formation in the liver, and serving to lower the concentration of blood glucose back to normal.

How do Pancreatic beta Cells Secrete Insulin?

Pancreatic beta-cells sense nutrients to maintain blood glucose homeostasis by secreting insulin. The canonical pathway of the pancreatic beta-cell insulin secretion is also referred to as glucose-stimulated insulin secretion (GSIS). It has three defined steps:

1) Glucose Entry

Glucose enters the beta cell through GLUT2 transporters via facilitated diffusion and is phosphorylated to produce glucose-6-phosphate via the rate-limiting enzyme glucokinase (Matschinsky, Glaser, & Magnuson, 1998). Rodents primarily express the high K_m (11.2mM) GLUT2 transporters, while human islets and beta cells have been shown to express GLUT1 (Km 6.9mM) and GLUT3 (Km 1.3mM)(Gould & Holman, 1993; Henquin, Dufrane, & Nenquin, 2006).

While the expression of the GLUT transporters in humans and rodents are disparate, its has been shown that expression and influence of glucokinase is similar making it a central control point for GSIS(De Vos et al., 1995; Matschinsky et al., 1998). Glucokinase has a rate-limiting step in glucose metabolism (Z. Fu, Gilbert, & Liu, 2013). Unlike its isozyme, Hexokinase, glucokinase has 2 unique features: a lower affinity for glucose (K_m 6mmol/L), which places it in an ideal physiological range (4-10mmol/L), as well as a lack of

product inhibition, enabling it to serves as a glucose sensor (Z. Fu et al., 2013; Matschinsky, 1996).

2) Glucose Oxidation

Glucose is further metabolized to pyruvate via glycolysis and enters the TCA cycle in the mitochondria to fuel the synthesis of ATP by oxidative phosphorylation. The increase in cellular ATP/ADP ratio leads to the closure of the ATP-sensitive potassium channels, which results in the depolarization of the plasma membrane (F. M. Ashcroft, 2006; F. M. Ashcroft, Harrison, & Ashcroft, 1984). Upon depolarization, voltage-gated calcium channels are opened, leading to insulin secretion (Wollheim & Sharp, 1981). Mitochondrial oxidative function is central to this process, as mitochondrial defects were shown to disturb normal beta-cell function (Anello et al., 2005; Maechler & Wollheim, 2001; Stiles & Shirihai, 2012).

3) Membrane Depolarization and Exocytosis

The K_{ATP} channel serves as a link that senses the increase in glucose via an increase in the ATP/ADP ratio and the subsequent electrical excitation of the cell signaling the release of insulin. The K_{ATP} channel is open under low glucose conditions, allowing efflux of K down its gradient and keeping the cell in a hyperpolarized state. After a meal, under high glucose conditions, the increase in

the ATP/ADP ratio leads to the closure of the K_{ATP} channel consequently leading to the depolarization of the cell and secretion of insulin. Gain of function or loss of function mutations in this channel may result in diabetes or hyperinsulinism. Also, the SUR1 protein, a regulatory component of the K_{ATP} channel which senses changes in ATP and ADP, is the target of sulfonylurea drugs that stimulate insulin secretion by causing closure of the K_{ATP} channel thereby improving glycemic control(K. Bennett, James, & Hussain, 2010).

Depolarization & Voltage-gated Calcium Channels

The beta cell is electrically inactive at a resting membrane potential of approximately -70mV when the concentrations of glucose are less than 5mM. As the glucose concentration increases, the cell is depolarized and action potentials start to fire (threshold of -55mV to -50mv) in an oscillatory manner in the range of 6-17mM of glucose (Rorsman, Braun, & Zhang, 2012). With the advent of the patch-clamp method, it was possible to characterize the different ion channels involved in the electrically excitable beta cell. It was found that the membrane resistance increases as a result of the closure of the K_{ATP} channel leading to the opening of the T-type Ca^{2+} channels, which respond to a low threshold potential (activated by low change in voltage, -60mV). This subsequently activates the Na^{+} and L-type Ca^{2+} channels that correspond to the fast but brief upward stroke of the action potential. The activation of the P/Q Ca^{2+} channels contributes to the

peak of the action potential and triggers exocytosis of insulin vesicles. Repolarization of the action potential is a result of the activation of the high conductance K^+ channels (BK), which sense the increase in intracellular calcium and open, releasing the positively charged potassium ions thereby helping to reset the cells membrane potential(Rorsman et al., 2012).

Exocytosis of Insulin

Insulin secretion occurs in a regulated biphasic manner. The first phase of secretion is termed the readily releasable pool as it involves predocked granules that respond to the KCl and non-nutrient secretagogues (Gembal, Gilon, & Henquin, 1992; Z. Wang & Thurmond, 2009). The second phase of insulin release, however, responds to the change in glucose concentration and has a much higher impact, releasing 4-50 granules per cell per minute over a long period of time. The second phase of insulin release involves a rearrangement of the F-actin filaments, allowing the insulin granules to fuse with the plasma membrane in a SNARE dependent manner in which VAMP2, a v-SNARE, interacts with the syntaxins on the plasma membranes (t-SNAREs) in order to mediate the docking, fusion, and release of the insulin granules (Z. Wang & Thurmond, 2009). Impairment in this regulated process of both first and second phase has been associated with Type 2 Diabetic patients. This is especially important in the second phase of insulin release as the first phase only accounts

for approximately 1% of total granule insulin release, while the second phase is sustained for longer, and accounts for a larger total magnitude (Barg, Eliasson, Renström, & Rorsman, 2002). As insulin granules are synthesized, they are stored for days awaiting a signal for release. During that time the older, aged granules are subjected to degradation. It has recently been shown that the secretion of insulin granules occurs in an age-dependent manner. Younger insulin granules (less than 48 hours) are more likely to be released first (Hou et al., 2012; Michael, Xiong, Geng, Drain, & Chow, 2007).

B-cell Dysfunction in Diabetes Progression

Progression of diabetes is recognized to be a consequence of increased in insulin resistance and impairment of beta-cell function (Kahn, 2003). A strong association between obesity and development of T2D has been established (Golay & Ybarra, 2005; N. T. Nguyen, Nguyen, Lane, & Wang, 2011a). The majority of diabetics are obese, while the opposite does not hold true. This observation raised the question of investigating the relationship of fat accumulation to changes in insulin sensitivity. It has been shown that accumulation of visceral fat is the primary correlate to insulin sensitivity (Cnop et al., 2002; Kahn, 2003). In the progression of individuals from normal lean subjects, to obese individuals, to development of impaired glucose tolerance and ultimately diabetes, there is a concomitant rise in the levels of basal insulin

secretion from pancreatic beta cells (Pories & Dohm, 2012). Indeed, one of the first changes is hypersecretion of insulin in the fasting/basal state observed in the prediabetic obese, hyper-lipidemic state. Hypersecretion of insulin can indeed lead to insulin resistance either by downregulation or impaired sensitivity of insulin receptors in the peripheral tissues (Soman & DeFronzo, 1980). Insulin not only regulates blood glucose levels, but is also an anabolic hormone, thereby promoting fat storage leading to weight gain (Dimitriadis, Mitrou, Lambadiari, Maratou, & Raptis, 2011). Increased circulating lipids further exacerbate beta cell function by promoting basal hyperinsulinemia. Beta cell failure ultimately ensues when beta cells can no longer compensate for changes in peripheral tissue insulin sensitivity (Prentki & Nolan, 2006).

Evidence for Hyperinsulinemia as Primary Defect in Diabetes

Hyperinsulinemia (HI) is thought to represent a compensatory response to peripheral insulin resistance. Beta cell compensation ultimately leads to beta cell failure, in which beta cells do not secrete a sufficient amount of insulin in response to an acute nutrient challenge(Nolan, Leahy, Delghingaro-Augusto, Moibi, Soni, Peyot, et al., 2006a; Prentki & Nolan, 2006). During diabetes progression, there is a reduction in beta cell mass and/or differentiation of beta cells leading to functional loss and insulin dependence (Weir & Bonner-Weir, 2004). However, there is no conclusive evidence to support this hypothesis.

Multiple knockout models of insulin resistance have failed to manifest development of HI (Blüher et al., 2002; Brüning et al., 1998; Okamoto et al., 2004). While it is true that a hyperbolic relationship between insulin sensitivity and insulin secretion exists, evidence indicates that hypersecretion of insulin may be the initial defect in progression of T2D(Corkey, 2012a; Retnakaran et al., 2008). Multiple modes of chronic exposure to HI such as insulin infusion in humans, hyperglycemic clamps or transgenic animal models, demonstrate development of peripheral insulin resistance(P. D. Miles et al., 1998; Rizza, Mandarino, Genest, Baker, & Gerich, 1985; Shanik et al., 2008). Inhibition of insulin secretion with K_{ATP} channel agonists such as diazoxide and NN414 improves insulin sensitivity in both human and animal studies further reinforcing this hypothesis(Alemzadeh, Fledelius, Bodvarsdottir, & Sturis, 2004; Alemzadeh, Langley, Upchurch, Smith, & Slonim, 1998; Carr, Brand, Bodvarsdottir, Hansen, & Sturis, 2003; Zdravkovic, Kruse, Rost, Møss, & Kecskes, 2007; Zdravkovic et al., 2005). Additionally, an etiologic role for HI has been shown in certain ethnic groups such as African Americans and Pima Indians which have a higher susceptibility for development of T2D that is unaccounted for by insulin resistance(Aronoff, Bennett, Gorden, Rushforth, & Miller, 1977; Arslanian, Saad, Lewy, Danadian, & Janosky, 2002; Weyer, Hanson, Tataranni, Bogardus, & Pratley, 2000). Remission of T2D post roux-en-y gastric bypass surgery in which HI is normalized independent of peripheral insulin resistance or weight loss is yet another interesting correlation suggesting a need to further dissect mechanisms

of HI(Camastra et al., 2011; Reed et al., 2011). In fact, prevention of fasting hyperinsulinemia by ablation of pancreatic beta cell specific insulin gene *Ins1* is protective from diet-induced obesity (Mehran et al., 2012). It is currently unknown what mechanisms drive the hypersecretion of insulin in the beta cell. Thus, understanding mechanisms of basal hyperinsulinemia is critical and could indicate an early point of intervention at the pre-diabetic state to reverse development of T2D and prevent beta cell failure.

Regulation of Basal Insulin Secretion

Very little research has investigated the mechanisms underlying hypersecretion of insulin in the basal state. Boden and colleagues explored the possibility that basal FFAs potentiate basal insulin secretion by acutely lowering FFAs via administration of nicotinic acid, which prevents TG lipolysis by adipose tissues (Boden, Chen, & Iqbal, 1998) (Kamanna & Kashyap, 2008). This results in 25-33% reduction in basal secretion rate. This is contradictory to what is observed during fasting, when fatty acids are markedly high and insulin secretion is at its lowest. Evidence suggests that this may be due to signals that may influence the threshold required to activate calcium channels (Erion, Berdan, Burritt, Corkey, & Deeney, 2015). It is noteworthy to mention that glucose stimulation of insulin secretion in the fasted stated necessitates presence of free fatty acids (Stein et al., 1996).

Treatment of islets with oleate increased insulin secretion at basal glucose (Erion et al., 2015) and was shown to have a marked reduction in Glucose-6-phosphate content, as a result of increased PFK activity. This is thought to occur from increased PFK expression (Clarke, 1994; Roche et al., 1997) or due to a shift of increased glucose utilization from glucokinase to hexokinase (Liang, Buettger, Berner, & Matschinsky, 1997), since increased conversion of LC-CoA to malonyl Co-A inhibits beta-oxidation through CPT-I (Brun et al., 1996; S. Chen et al., 1994). Triacsin C, a fatty acyl Co-A synthetase inhibitor, restored G6P content, prevented the oleate induced increase in PFK activity, and reduced basal insulin secretion, implicating a signaling role for LC-CoA (Y. Q. Liu, Tornheim, & Leahy, 1998).

Monoglycerides were also shown to acutely stimulate basal insulin secretion via increased ROS production, without altering intracellular calcium or oxygen consumption (Saadeh et al., 2012). This occurred in a K_{ATP} independent manner, as treatment with diazoxide blunted GSIS, but had no effect on basal. Hence, monoglycerides may function as an *in vitro* tool to examine mechanisms of insulin secretion under basal glucose conditions.

The Role of Excess FFA in beta Cell Dysfunction in Pre-diabetes and T2D

Lipids and Insulin Secretion

Observations from early studies of intravenous lipid infusion in dogs indicated that elevated levels of plasma free fatty acids stimulate insulin secretion (Crespin, Greenough, & Steinberg, 1969; Madison, Seyffert, Unger, & Barker, 1968) To establish a direct effect on the pancreas, Crespin et al directly infused sodium salts of oleic, linoleic, lauric, or palmitic acid into pancreatic arteries of dogs at physiological concentrations which equipotently stimulated insulin secretion without altering peripheral FFA levels (Crespin, Greenough, & Steinberg, 1973). The high oxidation rates fatty of palmitate and oleate revealed them to be important fuels for pancreatic beta cells in the fasting state, although no strong effect on insulin secretion is observed (Berne, 1975).

Fatty acids acutely amplify insulin secretion but chronically are detrimental to beta cells function. High fat diet mice were shown to have reduced glucose stimulated insulin secretion and reduction in pro-insulin mRNA levels (Capito, Hansen, Hedeskov, Islin, & Thams, 1992). Correspondingly, chronic incubation with fatty acids was shown to inhibit GSIS, an effect that was prevented by inhibition of fatty acid oxidation (Elks, 1993) (Y. P. Zhou & Grill, 1994). It is well established that there is an increased level of circulating lipids in the pre-diabetic

obese state (Paolisso, Tataranni, et al., 1995b) (Jensen, Haymond, Rizza, Cryer, & Miles, 1989)

Development of the Lipotoxicity Hypothesis

Unger first developed the concept of lipotoxicity in the early 1990's (Unger, 1995). This idea followed evidence from defects produced by FFAs that reflect/represent the pathological changes observed in Type 2 diabetes, namely hyperinsulinemia, impairment of GSIS, and development of insulin resistance (Y. Lee et al., 1994). ZDF pre-diabetic rats exhibited higher plasma FFA, triglyceride content, and basal insulin prior to onset of hyperglycemia due to loss of GSIS and GLUT2, an effect that was reversed by pair feeding with lean littermates (Y. Lee et al., 1994). The increase in basal and reduction in GSIS are similar to those observed by in vitro incubation of islets with long chain fatty acids (Y. P. Zhou & Grill, 1994). The impairment of GSIS upon long-term exposure of pancreatic islets to palmitate was shown to result from a decrease in PDH activity and an increase in PDH Kinase, which inactivates PDH by phosphorylating it (Y. P. Zhou & Grill, 1995).Influence of fatty acids on insulin secretion is time-dependent; fatty acids are stimulatory upon short-term exposure in a manner that is dependent upon glucose concentration, but inhibitory to GSIS upon chronic intralipid infusion (Paolisso, Gambardella, et al., 1995a; Sako & Grill, 1990). *In vitro* findings are analogous, whereby acute treatment of beta cells with fatty

acids is stimulatory whereas chronic treatment is inhibitory for GSIS (Bollheimer, Skelly, Chester, McGarry, & Rhodes, 1998; Elks, 1993; Gravena, Mathias, & Ashcroft, 2002). The chain length and degree of unsaturation of fatty acids also have important properties since long chain and saturated fatty acids are more potent for GSIS (Stein et al., 1997). Fatty acids are thought to amplify insulin secretion acutely via fatty acid receptors such as GPR40 which may serve to increase fatty acid cycling (Itoh et al., 2003). Knockout of GPR40 has shown mixed results however, and its role remains controversial since some studies show a protective role, while others indicate no protection from high fat diet (HFD) (Poitout et al., 2010). Chronically, fatty acids are detrimental to GSIS via build up of toxic lipid metabolites, ceramides, and DAG (Kelpe et al., 2003), in addition to reduced insulin gene transcription (Ritz-Laser et al., 1999) and sustained oxidative stress (Hauck & Bernlohr, 2016; Ly et al., 2017).

Saturated vs. Unsaturated Fatty Acids

Palmitate lipotoxicity results from the beta cells' inability to shuttle it into triglycerides for storage (Listenberger et al., 2003). Co-supplementation of unsaturated fatty acids, such as oleate, has been shown to rescue from the apoptotic effects of excess palmitate (Listenberger et al., 2003) (Maedler et al., 2001). Moreover, palmitate elicits ER stress and results in striking morphological changes in ER, mitochondria, and Golgi (Karaskov et al., 2006) Desaturation in

beta cells is protective, as co-treatment with unsaturated fatty acids or overexpression of desaturases such as SCD1 ameliorates palmitate induced damage (Busch et al., 2005; Thörn, Hovsepyan, & Bergsten, 2010). From a physiological standpoint, it is more prudent therefore to utilize lipids that are physiologically more relevant either by supplementing beta cells with oleate, a lipid they are capable of handling, or of a combination of saturated and unsaturated fatty acids.

Although unsaturated fatty acids such as oleate may be protective from inducing ER stress, they still play a role in contributing to beta cell dysfunction. Chronic infusion of oleate followed by application of hyperglycemic clamps results in impaired GSIS which is mediated by oxidative stress, an effect that is restored by treatment with antioxidants (Oprescu et al., 2007).

Fatty acids amplify insulin secretion in the presence of glucose, while glucose excess is necessary for excess lipids to be toxic (Nolan, Madiraju, Delghingaro-Augusto, Peyot, & Prentki, 2006b) (Poitout et al., 2010) This suggests presence of coupling factors that mediate the effects of glucolipotoxic conditions (Prentki & Corkey, 1996).

Increased tissue triglyceride content is correlated with the increased insulin resistance and hyperinsulinemia of obesity (Koyama, Chen, Lee, & Unger,

1997) Pre-diabetic islets display an increased lipogenic capacity which may constitute a compensatory response that exacerbates beta cell dysfunction (Y. Lee et al., 1997)

Role of Mitochondria in Lipid-induced beta Cell Dysfunction

Mitochondria are dynamic double membrane bound organelles that play central role in ATP production, and repeatedly undergo fission and fusion events allowing them to recover, share proteins and solutes, and increase their metabolic efficiency. Fusion triggers a fission event, producing two distinct daughter mitochondria, segregated by their difference in membrane potentials. One daughter mitochondrion is hyperpolarized and undergoes a subsequent fusion event, while the other daughter mitochondrion is depolarized and is targeted to undergo autophagy unless it recovers and repolarizes (Twig et al., 2008). It is currently unknown mechanistically how this phenomenon occurs. Depolarized and fragmented mitochondria have been associated with a reduced respiratory capacity, increased oxidative damage and impaired insulin secretion, which is restored by shifting mitochondrial dynamics to a more fused state (Molina, Wikstrom, Stiles, Las, Mohamed, Elorza, Walzer, Twig, Katz, Corkey, & Shirihai, 2009a). As the primary fuel sensors in the cell an understanding of changes in mitochondrial bioenergetics function is crucial to understanding one aspect of the pathophysiology of diabetes.

Moreover, mitochondria play a particularly central role in the beta cell by integrating different types of fuels and producing metabolic signals that can be used to trigger secretion (Maechler & Wollheim, 2001). Beta cells devoid of mtDNA lose the ability to couple metabolism of nutrients to insulin secretion, highlighting a essential role for mitochondria in beta cells (Soejima et al., 1996) (Kennedy, Maechler, & Wollheim, 1998) (Tsuruzoe et al., 1998). Metabolites are broken down and electrons are stripped by the electron transport chain driving an increase in the mitochondrial membrane potential or proton motive force. The proton motive force is consumed either by the production of ATP (via increased energy demand) or is dissipated by proton leak. These cycles of exporting protons out via ETC activity and their re-entry via ATP synthase and/or leak determine mitochondrial oxygen consumption. The degree to which ATP production and oxygen consumption are coupled reflects bioenergetic efficiency.

An intriguing and unique feature of beta cell mitochondria is their inherent bioenergetic inefficiency (Affourtit & Brand, 2009) (Wikstrom et al., 2012). This high inefficiency might serve beta cells to act as fuel sensors, and therefore metabolize and sense nutrients irrespective of their ATP demand.

Characterization of T2D human islets display marked changes in mitochondrial features such as fragmented and hypertrophied morphology and a

reduction in membrane potential (Anello et al., 2005). *In vitro* studies of excess nutrient treatment of beta cells with lipotoxicity and glucolipotoxicity revealed that the increase in mitochondrial fragmentation results from a reduction in fusion. This fragmentation was primarily driven by the excess of non-physiological fatty acid concentration of palmitate (lipotoxicity) with a synergistic effect upon glucolipotoxicity (Molina, Wikstrom, Stiles, Las, Mohamed, Elorza, Walzer, Twig, Katz, Corkey, & Shirihai, 2009b). Long term exposure to excess palmitate has been associated with increased mitochondrial proton leak and excess ROS (Carlsson, Borg, & Welsh, 1999). This hinted at the possibility that identification of the source of leak may be a possible therapeutic target for diabetes.

Mechansims for beta Cell Bioenergetic Inefficiency and its Relevance for T2D and beta Cell Dysfunction Induced by Lipids

The mitochondrial proton motive force is primarily built to generate an energy potential driving the synthesis of ATP. Dissipation of the proton gradient via mechanisms that do not result in ATP production leads to mitochondrial uncoupling. Mitochondrial uncoupling may results from various changes in exchangers, ion channels etc. In brown adipose tissue, the main driver of leak/energy dissipation was discovered to be UCP1 (Nicholls & Locke, 1984). Researchers subsequently identified UCP homologs in other tissues. It was

strongly believed in the field that UCP2 is the main contributor of the leak in beta cells.

The discovery of UCP2 by Fleury et al in 1997 generated a shift in the diabetes field, as it was proposed that UCP2, which shares 59% sequence homology with UCP1, is a ubiquitously expressed thermogenic protein. UCP2 was determined to have a larger effect on mitochondrial membrane potential when expressed in yeast, while gene mapping in mouse and humans linked UCP2 to regions linked to hyperinsulinemia and obesity. UCP2 was hypothesized to play a role in obesity by regulation of body weight via its effect on energy expenditure. This prompted research towards characterization of this novel diabetes associated protein (Fleury et al., 1997).

Overexpression Studies

During that time, leptin was shown to induce UCP2 mRNA expression, increase enzymes involved in FA oxidation (CPT1 and ACO), while also decreasing intracellular lipid/triglyceride content (Y. T. Zhou et al., 1997). However, ZDF islets, which are insensitive to leptin, have reduced UCP2 expression, increased TG content, and reduced GSIS. Introducing UCP2 to ZDF islets surprisingly rescued GSIS by promoting insulin gene expression, while displaying increased glucose and fatty acid oxidation, reduced ATP content, and

increase in ATP:ADP ratio, with no change in TG content (M. Y. Wang et al., 1999).

A contradictory finding was observed however, when UCP2 overexpression was performed in normal rat islets. In this study, an inhibitory effect on GSIS was observed upon overexpression of UCP2 without an effect on insulin content (Chan et al., 1999). A follow-up study by the same group found that overexpression of UCP2 in normal rat islets decreased ATP content by 50%. The same study showed that rats on 3 weeks HFD increased expression of UCP2 in islets and increased *in vitro* basal insulin secretion from isolated islets, although there was no difference in *in vivo* fasting insulin secretion (Chan et al., 2001).

The role of UCP2 in reduction of GSIS upon chronic (72h) beta-cell exposure to excess FA oleate was also investigated. It was found that long term FA treatment results in increased UCP2 gene expression with concomitant reduction in cytosolic ATP/ADP ratio and plasma and mitochondria membrane potentials in response to glucose(Lameloise, Muzzin, Prentki, & Assimacopoulos-Jeannet, 2001).

However, beta cell specific overexpression of UCP2 in transgenic mice and INS-1 cells, did not reveal any changes in plasma glucose or insulin, GSIS,

mitochondrial membrane potential, oxygen consumption, or ATP/ADP ratio. The authors conclude that evidence suggests that UCP2 does not have a role in proton leak and that perhaps it has a protective role from the observation that cytokine-induced ROS production is reduced (Produit-Zengaffinen et al., 2006).

The role of UCP2 in antioxidant stress defense was also explored. This study found a transient time dependent increase in UCP2 mRNA expression upon acute H_2O_2 induced oxidative stress, while the contrary was observed upon treatment with antioxidants (Vit E and selenite) (Li, Skorpen, Egeberg, Jørgensen, & Grill, 2001). It was subsequently shown that transient overexpression of UCP2 plays a protective role albeit modest from H_2O_2 induced oxidative stress (Li et al., 2001). It is noteworthy to mention that INS1 cells detected higher UCP2 expression than pancreatic islets (Li et al., 2001).

Knockout Studies

Generation of a UCP2 knockout mouse model confirmed UCP2 is a negative regulator of insulin secretion. Ob/ob mice which exhibit increased UCP2 mRNA and protein, were rescued by UCP2 KO displaying increased insulin secretion and improved glucose tolerance (C.-Y. Zhang et al., 2001). Subsequently, UCP2 KO mice under HFD were shown to promote better glucose

tolerance, attributed to improved insulin secretory capacity due to an increase in pancreatic beta cell mass and increased insulin content (Joseph et al., 2002).

Evidence Against UCP2 Leak Role

In marked contrast to previous UCP KO studies, Pi and colleagues showed in 3 independent mouse strains that KO of UCP2 induces oxidative stress while drastically impairing beta cell function with a marked reduction in GSIS (Pi et al., 2009). These completely opposing findings were attributed to differences in genetic background, which results in a genetic bias between KO and WT littermates, highlighting the importance of maintaining a congenic strain to avoid misinterpretation of findings (Pi & Collins, 2010).

A beta cell specific UCP2 KO suggested a role for UCP2 in regulation of intracellular ROS, while substantial evidence indicated against an uncoupling role for UCP2. This was achieved by showing that lack of UCP2 did not have an effect on islet ATP content, glucose stimulated respiration, or uncoupled respiration, even though they had a higher basal OCR. The animals were surprisingly glucose intolerant. This was attributed to an increased alpha cell mass, increased glucagon content, and increased glucose induced glucagon secretion which the authors speculate is a result of the increased islet ROS content (Robson-Doucette et al., 2011).

Permeability Transition Pore as a Physiological Leak Mechanism

Identification/Characterization/Regulation

It has long been recognized that mitochondrial calcium accumulation induces an increase in mitochondrial volume, reflected by increased light scattering of isolated mitochondria(Crofts & Chappell, 1965) (Chappell & Crofts, 1965). This observation of calcium induced permeability transition led researchers to dissect the mechanisms by which this phenomenon occurs. Haworth and Hunter first showed that permeability transition occurs via a non-selective channel which allows solute flux of substrates up to 1.5kD (Haworth & Hunter, 1979), while Crompton revealed that the pore opening has a diameter of 2.3nm (Crompton & Costi, 1990). Calcium and oxidative stress synergistically induce pore opening (Crompton, Costi, & Hayat, 1987). Regardless of the size of the pore and its activation, this event would decrease mitochondrial efficiency, since opening of the pore results in dissipation of the proton motive force that is uncoupled to ATP production. However, it might likely impair mitochondrial respiratory function if uncontrolled. Evidence indicated permeability transition can be controlled and reversible, meaning that it can be a mechanism for regulation of bioenergetics efficiency and mitochondrial function. Numerous physiological factors, in addition to ROS and calcium, have been subsequently shown to modulate the probability of pore opening. Mitochondrial calcium overload (Wong, Steenbergen, & Murphy, 2012) (Sloan et al., 2012), depolarization (Bernardi,

1992), uncoupling (Petronilli, Cola, Massari, Colonna, & Bernardi, 1993), fatty acids (palmitate) (Petronilli et al., 1993), inorganic phosphate (Halestrap, 2009), ROS (Halestrap, Woodfield, & Connern, 1997; McStay, Clarke, & Halestrap, 2002), potentiate mPTP opening, while adenine nucleotides (ADP and ATP), bongkrekic acid (ANT inhibitor) (Peng, Straub, Kane, Murphy, & Wadkins, 1977), BSA (via FA sequestration), NADH (Hunter & Haworth, 1979) Mg^{2+}, CsA (Wong et al., 2012) and acidic pH (Halestrap, 1991) (Bernardi et al., 1992) prevent calcium induced permeability transition.

Importantly, sustained opening of the permeability transition pore (PTP) depolarizes mitochondria, since proton leak dissipates the proton motive force. Bioenergetic failure ultimately ensues as the combination of uncoupled respiration and reversal of complex V (ATP synthase) will result in ATP depletion (Bernardi, 1999) (Halestrap, Clarke, & Javadov, 2004). Unfolding of mitochondrial cristae, and eventually membrane rupture leads to release of cytochrome c and other apoptotic stimuli (Halestrap, 2009) (Doran & Halestrap, 2000) (Martinou & Green, 2001). However, this is not the only mechanism for cytochrome c release as evidence suggests this may occur via pore dependent and independent mechanisms(Alcalá, Klee, Fernández, Fleischer, & Pimentel-Muiños, 2008; Doran & Halestrap, 2000)

Structure of the Permeability Transition Pore

While characterization of factors that regulate the pore has been established, deciphering how the structural components of the PTP are interconnected has been challenging and remains an open question (Giorgio, Guo, Bassot, Petronilli, & Bernardi, 2017). Evidence for an inner mitochondrial membrane ion channel was achieved in 1987 by Sorgato et al, in which patch-clamping of mitoplasts suggested this could be an inherent uncoupling protein (Sorgato, Keller, & Stühmer, 1987). It was subsequently proposed that the Mitochondrial Mega Channel (MMC) was in fact the mitochondrial permeability transition pore (mPTP), since it displayed similar response to elements that are known influence mPTP activity such as pH, Ca^{2+}, and cyclosporine A (CsA) (Szabó & Zoratti, 1992) (Bernardi et al., 1992). Initially, the prevalent hypothesis in the field postulated that the pore consisted of multiple proteins including the voltage dependent anion channel (VDAC) in the outer membrane, adenine nucleotide translocator (ANT) and the mitochondrial phosphate carrier in the inner membrane, hexokinase (linked to VDAC in the cytosol), Cyclophilin D (CypD) in the matrix, and creatine kinase in the inner membrane space, among other associated regulatory proteins (Zamzami & Kroemer, 2001) (Giorgio et al., 2017). However, genetic ablation of VDAC (Baines, Kaiser, Sheiko, Craigen, & Molkentin, 2007), ANT {Kokoszka:tk}, mitochondrial phosphate carrier (Gutiérrez-Aguilar et al., 2014), benzodiazepine receptor (Šileikytė et al., 2014), still results in permeability transition, indicating these are dispensable.

Although it was known that CsA prevents permeability transition, Bernardi's group was the first to show that this was via interaction with CypD (Nicolli, Basso, Petronilli, Wenger, & Bernardi, 1996). They subsequently provided the first evidence that CypD associates with the lateral stalk of the ATP Synthase, in which Pi enhanced, while CsA prevents, binding in a calcineurin independent manner (Giorgio et al., 2009). However, CypD was identified as only a regulator of PTP, since ablation of CypD necessitates a higher calcium threshold to activate permeability transitions (Basso et al., 2005). Furthermore, isolated mitochondria from mouse KO of CypD do not undergo permeability transition or swelling (Baines et al., 2005). In vivo, these mice are protected from ischemia reperfusion injury which is characterized by mitochondrial Ca^{2+} overload and oxidative stress (Baines et al., 2005). It is important to emphasize however, that although permeability transition requires a two-fold increase of Ca^{2+} in $PPIF^{-/-}$ mitochondria, CypD is not involved in other factors that sensitize PTP opening such as membrane potential, oxidative stress, and adenine nucleotides.

Cyclophilin D is a peptidyl prolyl *cis-trans* trans-isomerase that possesses chaperone properties and is involved in protein folding (Elrod & Molkentin, 2013). The full length protein, including the mitochondrial targeting sequence is 22kDa, while the matrix targeted, cleaved isoform is 18kDa (N. Johnson, Khan, Virji,

Ward, & Crompton, 1999) The crystal structure has revealed that cyclophilin D contains eight β-strands, two α-helices, and one 310-helix (Kajitani et al., 2008). While not much is known in terms of transcriptional regulation of CypD(Elrod & Molkentin, 2013), several modes of posttranslational modifications have been identified. Cyclophilin D has been suggested to be regulated by phosphorylation via glycogen synthase kinase 3β (GSK3β) (Xi, Wang, Mueller, Norfleet, & Xu, 2009), although this is disputed since it remains incompletely understood how kinases and phosphatases are regulated in the mitochondrial matrix(Elrod & Molkentin, 2013). It has also been proposed that Sirt3 may inhibit PTP via deacetylation of CypD at lysine 166(Hafner et al., 2010). Furthermore, S-nitrosylation of Cysteine 203 displays a protective effect by making cells immune to ROS-induced death and mitochondrial swelling. It is thought that this occurs from prevention of cysteine oxidation(T. T. Nguyen et al., 2011b).

Bernardi and colleagues introduced a revised model of the PTP in which components have been simplified down to dimers of the ATP synthase and cyclophilin D. The ATP synthase assembles into zipper-like dimers, where CypD associates with the lateral stalk of the ATP synthase pore(Giorgio et al., 2009). Interaction was shown to be regulated by CsA, as it dissociates CypD and increases catalytic activity of the ATP synthase(Giorgio et al., 2009). Subsequent work via immunoprecipitation of individual b, c and OSCP subunits of the peripheral stalk revealed the OSCP subunit as the binding partner of

CypD(Giorgio et al., 2013). Reconstitution of dimers into lipid bilayers showed that ATP synthase can form a conductance channel that is sensitive to benzodiazepine 423 (Bz-423) which sensitizes the pore to Ca^{2+} (Giorgio et al., 2013). Silencing OSCP was found to increase PTP sensitivity Ca^{2+} to as well (Giorgio et al., 2013). While the simplified model of the ATP synthase dimers and CypD is fairly accepted, it remains to be determined by what mechanism the dimers form and how PTP opening occurs. Permeability transition was proposed to occur via the c-ring (Bonora et al., 2013) (Alavian et al., 2014). However, the Walker lab demonstrated that deletion of the c-ring still allowed calcium induced permeability transition (He et al., 2017b). Simulation models also dismiss this theory, since simulations indicate that the c-ring cannot be in a hydrated state, eliminating the possibility of ion conductance (W. Zhou, Marinelli, Nief, & Faraldo-Gómez, 2017). The Bernardi lab has suggested a plausible mechanism. They have hypothesized that calcium binding in the F1 β-subunit induces a conformational change in the OSCP subunit, thereby mechanically inducing PTP opening as the lateral stalk is thought to be a hinge, whereby regulation occurs at a 100 Angstrom distance (Giorgio et al., 2017). This remains controversial, however, since two recent papers show contradictory findings regarding the roles of the OSCP and peripheral stalk in permeability transition (Antoniel et al., 2018) (He, Carroll, Ding, Fearnley, & Walker, 2017a).

Pathophysiological Roles of PTP

Sustained PTP opening results in a collapse of the mitochondrial membrane potential, loss of ATP production, equilibration of solutes, mitochondrial swelling and apoptotic release of cytochrome *c* (Bernardi, Rasola, Forte, & Lippe, 2015; Petronilli, Nicolli, Costantini, Colonna, & Bernardi, 1994). Considerable research has implicated dysfunctional PTP regulation in various diseases. PTP opening has been linked mechanistically to the damaging effects observed in ischemia-reperfusion injury of the heart (Baines et al., 2005; Griffiths & Halestrap, 1993; Piot et al., 2008; Sloan et al., 2012), muscle fiber loss in Ullrich congenital muscular dystrophy(Irwin et al., 2003; Sorato et al., 2014), as well as Duchenne muscular dystrophy(Reutenauer, Dorchies, Patthey-Vuadens, Vuagniaux, & Ruegg, 2008). Neurological diseases, such as Alzheimer's, in which beta amyloids augment PTP induction (Du et al., 2008), and multiple sclerosis (Forte et al., 2007) also implicate PTP opening induced damage. Excessive PTP opening also drives diet-induced insulin resistance in mice (Taddeo et al., 2014). Knockout of CypD or use of PTP inhibitors is profoundly protective in all of these pathologies, exposing the PTP as an important therapeutic target.

In contrast, some cancer cells evade cell death by a reduction of pore opening resulting from desensitization (Bernardi et al., 2015). Hexokinase II

promotes survival of these cells, not only through its antioxidant function (Pantic et al., 2013), but also via its interaction with the OMM (Engelman, 2009), which maintains the PTP in a closed conformation (Chiara et al., 2008).

Physiological Role of PTP

While the PTP has been well characterized in terms of its damaging effects in diseased states and induction of cell death, its normal physiological role has not been extensively characterized and remains an open question. Evidence indicates that the PTP may undergo flickering, a low-conductance state of opening, which enables transmission of small ions such as H^+, K^+, and Ca^{2+}, but not larger solutes such as sucrose (Ichas, Jouaville, & Mazat, 1997). Flickering allows the propagation of Ca^{2+} waves, much like action potentials in excitable cells. The rapid transients are reflected by rapid changes in membrane potential, where depolarization is associated with increases in matrix calcein, an indication of PTP opening (Hüser, Rechenmacher, & Blatter, 1998). This phenomenon of calcium induced calcium release, is observed in isolated mitochondria as well as intact cells. Cyclosporin A prevents Ca^{2+} efflux in cardiomyocytes (Altschuld et al., 1992), indicating that the low conductance state of the PTP must have a physiological role (Ichas & Mazat, 1998). Ablation of CypD in the heart exacerbated pressure-overload-induced cardiac dysfunction and hypertrophy by increasing matrix calcium, cardiac dehydrogenase activity and the ratio of glucose to fatty acid oxidation (Elrod et al., 2010).

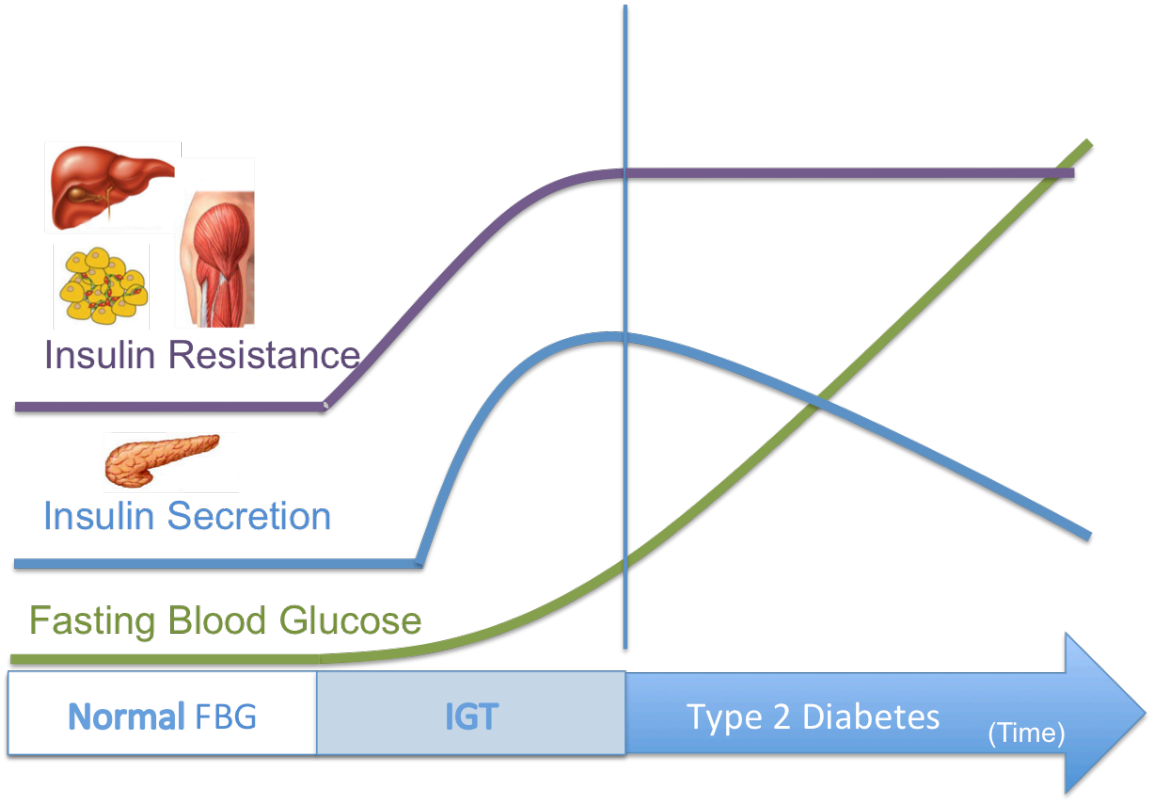

Figure 1.1. Conventional Model for Progression of T2D.

In the progression of T2D it is generally believed that insulin resistance of the peripheral tissues such as fat, liver, and muscle leads to compensatory basal hypersecretion of insulin. Insulin resistance alone does not cause diabetes. It is only when the compensatory increased beta cell workload ultimately results in beta cell failure that hyperglycemia ensues and diabetes occurs.

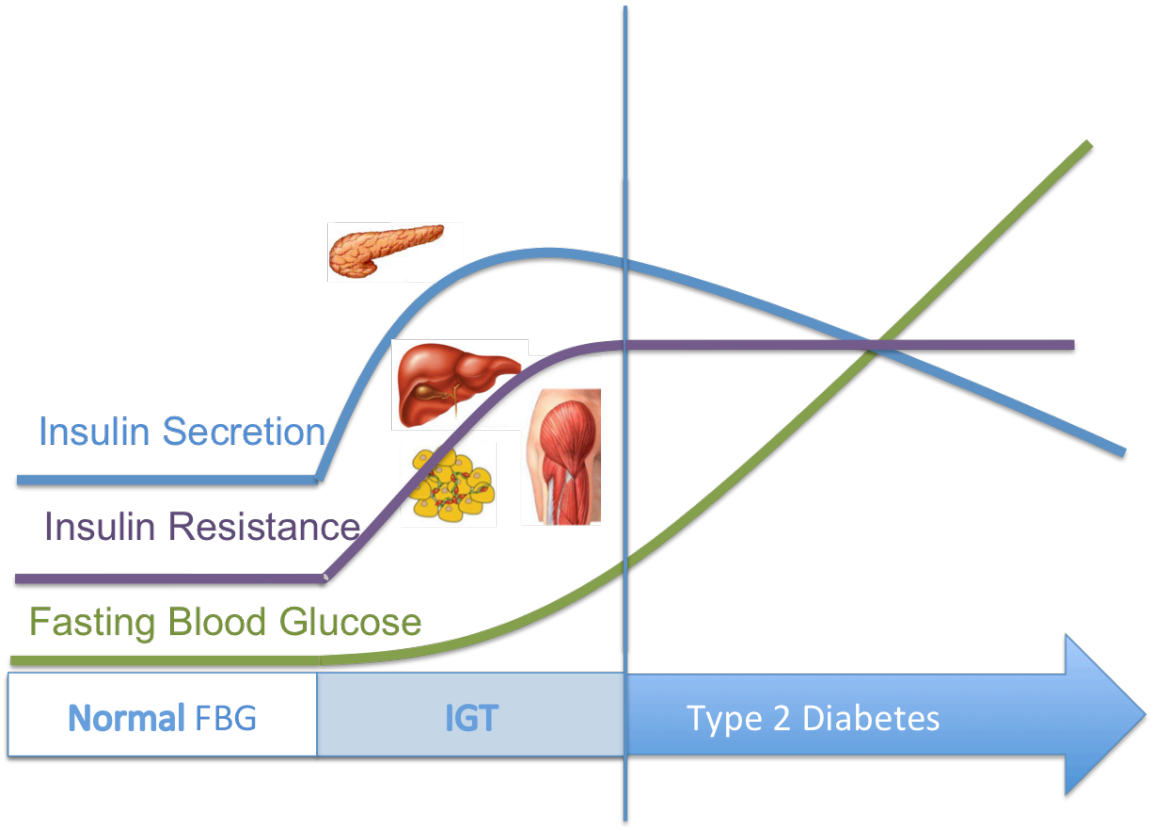

Figure 1.2. Alternative Model for T2D Progression: Basal Hyperinsulinemia Leads to T2D.

Excess nutrients result in basal hypersecretion of insulin in the obese. We propose an alternate model of diabetes progression, in which basal hyperinsulinemia may be a primary defect driving peripheral tissue insulin resistance, ultimately resulting in beta cell failure and hyperglycemia. It is unknown what mechanisms govern basal hypersecretion of insulin.

CHAPTER TWO: Methods

Cell Culture and Reagents

INS 832/13 cells were cultured in RPMI 1640 supplemented with 10%FBS, 10mM Hepes, 1mM Pyruvate, 50U/mL penicillin, 50g/mL streptomycin and 50uM 2-b-mercaptoethanol. Cells were cultured in either 11mM glucose or in 4mM glucose. A minimum period of 2 weeks in 4mM glucose was required for experiments of the 4mM glucose cells.

Animals

Islets were isolated from 12-14week old C57BL/J mice ordered from Jax labs. Mice were housed at University of California Los Angeles Animal Resources Facility. Animal care was in accordance with NIH guidelines and UCLA IACUC approval *(UCLA protocol #16-018).* Mice were housed in a 12hr/12hr light dark cycle and maintained at 68-72F and provided with water and food ad libitum until day of isolation. A maximum of 5 mice were housed per cage. Cyclophilin D mice were purchased from Jackson Laboratories (Stock 00907, B6;129-Ppif^{tm1Jmol}/J, generated by Dr. Jeffrey Molkentin, PMID: 15800627). WT and CypD KO littermate mice were produced from heterozygous breeding pairs, deriving from a male KO mouse and a female WT control

(Jackson Laboratories, Stock 101045, B6129SF2/J). Animals were sacrificed by isofluorane (UCLA) followed by cervical dislocation.

Genotyping

Mouse genotyping was outsourced to Transnetix. PCR of tail clips was performed by the protocol from Jax labs. The excised bands were 600bp for the mutant, and 850bp for WT. The following primers were used CTC TTC TGG GCA AGA ATT GC for (WT #8584), ATT GTG GTT GGT GAA GTC GCC (common #8585), GGC TGC TAA AGC GCA TGC TCC (mutant forward #8586)

Islet Isolation

Pancreatic islet isolations were performed as previously described (Wikstrom et al., 2013). After sacrificing the animals, an incision was made to expose the body cavity. The fat pads and viscera are gently moved down and towards the right, to expose the bile duct. Clamping the juncture of the bile duct and intestines is necessary to secure inflation of the pancreas. Islets of Langerhans were isolated by collagenase perfusion via the bile duct with RPMI media containing collagenase at a concentration of 1mg/mL. Perfusion via anatomical structures allows for better inflation of the pancreas and results in a higher islet yield. Pancreata were collected and left on ice for a maximum of one

hour. Islets were digested in 37°C water bath for 10min, after which they were washed 3x with RPMI media containing FA-free BSA at a concentration of 1g/100mL to stop digestion. Histopaque 1077 gradient centrifugation was performed to separate islets. Islets were subsequently collected and washed twice with FA-free BSA RPMI solution, after which they were picked and cultured overnight in beta cell media consisting of RPMI, 10%FBS, 50U/mL penicillin, and 50g/mL streptomycin. An average yield of 100 islets was obtained per mouse.

Insulin Secretion Assay- INS1

INS1 insulin secretion assays were performed in a static incubation on cells that have reached 70-80% confluence in 96-well black-sided clear bottom plates. Cells were pre-incubated in 2mM glucose RPMI containing 10%FBS, 10mM Hepes, 1mM Pyruvate, 50U/mL penicillin, and 50g/mL streptomycin. This was followed by a 30 minute pre-incubation in 2mM glucose Krebs at 37°C. Insulin secretion samples were collected after a subsequent 1 hour incubation at 37°C in either 2mM glucose Krebs or 12mM glucose Krebs for basal or glucose stimulated insulin secretion, respectively. Krebs solution consisted of 119mM NaCl, 20mM Hepes, 4.6mM KCl, 1mM $MgSO_4$, 0.15mM Na_2HPO_4, 0.4mM KH_2PO_4, 5mM $NAHCO_3$, 2mM $CaCl_2$, 0.5%BSA, pH 7.4.

Insulin Secretion Assay-Islets

Intact islets (6 islets/well) were pre-incubated in 3mM glucose Krebs solution for 30min in a 37°C H_2O bath. This was followed by a 45 minute incubation in either 3mM or 16.7mM glucose Krebs solutions for basal or glucose stimulated insulin secretion, respectively. Leucine/Glutamine, Oleate/Palmitate, MnTBAP, and Nim811 were added during these incubations at the concentrations indicated.

Insulin Measurements

An HTRF insulin assay kit was used to measure insulin secretion (CisBio, Bedford, MA). The assay consists of a FRET-based sandwich immunoassay with Europium Cryptate (Eu-K) and allophycocyanin (XL665) antibodies. Secretion samples were appropriately diluted in a 96-well plate after which 10µL of sample and 10µL of the antibody mix were dispensed into white 384-well plates (Greiner catalog # 784076) and incubated overnight at room temperature. A Tecan Infinite M1000 or Spark 10M plate reader was used to measure time-resolved fluorescence at emission wavelengths of 665nm (XL665) and 620nm (Eu-K). Insulin concentration of the samples was calculated from an insulin standard curve. Values that did not fall within the standard curve were excluded or rediluted and reassayed.

Western Blotting

Protein isolation was performed by washing islets with PBS on ice followed by addition of RIPA lysis buffer (Santa Cruz Biotechnology) with addition of 2% Triton-X-100 and protease inhibitors (Santa Cruz Biotechnology) for 5 minutes. Islet lysates were collected and continued to sit on ice for 30min. Lysates were then centrifuged at 12,000 rpm for 10min at 4C after which the supernatant was collected. A BCA assay (Thermo Fisher Scientific) was used to determine protein concentration. Samples were reduced and denatured by addition of β-mercaptoethanol and 4x NuPAGE LDS sample buffer (Invitrogen) followed by boiling at 95C for 10min. Samples were loaded on a 4-12% polyacrylamide gradient gel and a wet transfer to a PVDF membrane was performed overnight at 4C. The membrane was blocked with 5% BSA PBS solution containing 0.1% Tween-20 (PBST) for 1 hour after which it was incubated overnight at 4C in the primary antibodies indicated. For cyclophilin D the antibody used was mouse monoclonal cyclophilin F (Abcam, ab110324) at 1:1000 dilution. The membranes were then washed three times in PBST for 5 min and subsequently incubated in the appropriate anti-Rabbbit or anti-Mouse IgG secondary antibodies for 45min at room temperature. Membranes were again washed 3 times with PBST. A chemiluminescent Supersignal West Femto-Max Sensitivity Substrate (Cat. #34095 ThermoScientific) was used to detect protein signal. B-actin or GAPDH were used as loading controls to correct for

protein loading. Densitometry of changes in protein expression was performed in ImageJ software.

Preparation of Fatty Acids

Palmitate-BSA

Palmitate was complexed to BSA at a ratio of 4:1. Palmitic acid (Sigma) was dissolved in DMSO to make a final concentration of 400mM. The Palmitate-DMSO was then dissolved in a 6.7% fatty acid-free BSA RPMI 1640 no glucose solution at 45°C to produce a 10x stock of 4mM Palmitate(Las, Serada, Wikstrom, Twig, & Shirihai, 2011). Stocks were stored at -80°C. On the day of treatment, the solution was added to 1% FBS RPMI solution containing 50U/mL penicillin, 50g/mL streptomycin and 11mM glucose or as indicated. Solutions were then adjusted to pH 7.4, filtered and cells treated for indicated time periods.

BSA Control

For the BSA control, a 6.7% FA-free BSA RPMI solution containing 1% DMSO was made to prepare a 10x stock solution(Las et al., 2011).. Stocks were stored at -80°C. On the day of treatment, the solution was added to 1% FBS RPMI solution containing 50U/mL penicillin, 50g/mL streptomycin and 11mM glucose or as indicated. Solutions were then adjusted to pH 7.4, filtered and cells treated for indicated time periods.

Oleate-Palmitate BSA

Sodium palmitate (Sigma, cat. # P9767) and sodium oleate (Sigma, cat. # O3880) were complexed to Ultra FA-free BSA (Roche Applied Science, cat. # 03117405001) in a 150mM NaCl solution at 37°C at a FA:BSA ratio of 2:1 (1:1 ratio of oleate:palmitate) to produce a 10mM OP stock solution(Trudeau et al., 2016). The pH of the solutions was then adjusted to 7.4 using NaOH, filtered and stored at -20°C. The solutions were diluted in 1% FBS RPMI solution containing 50U/mL penicillin, 50g/mL streptomycin and 11mM glucose before treating INS1 cells or mouse islets for time indicated.

Oleate-FBS

Oleate 3.15 mol/L (Sigma O1008-5g) was dissolved in DMSO to a final concentration of 100mM or 200mM oleate-DMSO. The oleate-DMSO solution was then complexed to FBS serum at 57°C while vortexing to produce a 10x stock(Erion et al., 2015). 10x stock solutions of 1mM and 2mM oleate were stored at -80°C. For the control, FBS containing 1% DMSO was stored at -80°C as a 10x stock. Stock solutions were diluted in 0% FBS RPMI media containing either 4mM or 11mM glucose at the indicated concentrations.

Respirometry/O_2 Consumption

A seahorse extracellular flux analyzer XF96 (Seahorse Bioscience, Billerica, MA) was used to assess changes in oxygen consumption in INS-1 cells and intact islets as previously described (Wikstrom et al., 2012). For single islets respirometry, islets were transferred to 3mM glucose (basal) Seahorse media. Single islet respirometry was performed in PDL-coated 96-well XF plates. To maintain accurate OCR measurements, wells were plated with 1.5uL matrigel, to which islets were hand picked and carefully placed in center of the well. 6-8 wells of islets were seeded at a time. Matrigel was allowed to solidify for 2-3min, before addition of 150uL Basal SH media. Basal SH media was defined as 2.8mM glucose Seahorse XF Base Medium (Catalog # 102353-100) in 0.1%FBS. For port injections, various nutrients indicated were injected in port A, followed by final concentrations of 4.5 µM Oligomycin in port B, 1 µM FCCP in port C, and 2.5 µM Antimycin A in port D. All compounds were calculated such that final concentrations in well reflect total volume of well upon injection. Start volume was 150uL, and all injections were 25uL for a final volume of 250uL upon completion of the assay.

Quantification of Lipid Content

Changes in intracellular lipid content were determined by fixing cells with 4% paraformaldehyde (PFA) for 15 minutes, washing twice with PBS and staining with Bodipy (493/503) and Hoechst (to identify nuclei) for 15 minutes. An

Operetta high throughout imaging system was used to image 96-well black sided, clear bottom plates with preset filters for green and blue excitation. Prior to imaging every experiment, testing of optimal z-plane and exposure was determined to gain best focus and signal/noise ratio. Operetta STAR analysis software was used to determine nuclei to identify cell numbers, while Bodipy493 (green) signal/channel was used to determine cell area, lipid droplets intensity, number and area. Quantification of lipid droplet number per cell number was deemed to be the parameter most reflective of changes in lipid content.

Reactive Oxygen Species Measurements

CM-H2DCFDA (Invitrogen, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester), a molecular probe for hydrogen peroxide was used to detect changes in reactive oxygen species. Islets were dispersed and seeded in matrigel (BD Matrigel™ Basement Membrane Matrix) in 96-well cell culture plates. Dispersed islet cells were kept overnight in β-cell medium. The following day cells were loaded with 10 μM CM-H_2DCFDA (Invitrogen) for one hour at 3mM glucose. Cells were washed twice with Krebs buffer solution followed by 15min incubation. A Tecan Infinite M1000 plate reader was used to measure fluorescence emission detected at 520 nm at 37°C. Unstained wells were used as negative controls and positive controls were performed with tert-butyl hydrogen peroxide (Sigma).

CHAPTER THREE: Optimization of a β-cell Model of Glucolipotoxicity

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Introduction

Diabetes and obesity are associated with increased levels of free fatty acids in the circulation (Y. Lee et al., 1994). This suggests the hypothesis that overconsumption of nutrients leads to beta cell dysfunction. A hallmark of T2D is an impaired capacity of pancreatic beta cells to secrete insulin in response to nutrients. Different labs have employed similar but unique models of excess nutrients to investigate this hypothesis such as lipotoxicity (excess lipids), glucotoxicity (excess glucose), and glucolipotoxicity. The objective herein is to optimize an *in vitro* system of glucolipotoxicity mimicking the pathophysiological changes to beta cell function *in vivo* during diabetes progression. That is, excess fatty acid treatments that result in a reduction of glucose stimulated insulin secretion, while increasing basal insulin secretion (Sako & Grill, 1990; Y. P. Zhou & Grill, 1994). This would provide a system whereby the mechanisms resulting in beta cell dysfunction could be further dissected and characterized.

Results

Optimization of Palmitate Lipotoxicity Model

Free fatty acids are insoluble in aqueous solutions and therefore are complexed to BSA to increase solubility. This mimics the process by which fatty acids are normally transported through the circulatory system on albumin. Our lab has previously established a protocol in which palmitate is complexed to BSA at a FA:BSA ratio of 4:1 to make a 10x stock solution.(Las et al., 2011). This protocol is technically challenging and has proved difficult to reproduce, since it has not been optimized and there is variability in batch-to-batch preparations. The concentration of 0.4mM palmitate at a 4:1 ratio of FA:BSA is expected to reduce glucose stimulated insulin secretion (GSIS) as previously reported (Las et al., 2011; Trudeau et al., 2016). In Figure 3.1A the clonal rat beta cell line INS1 was cultured in 11mM glucose and concomitantly treated with the respective concentrations of palmitate indicated for 16-24 hours, after which an insulin secretion assay was performed. However, as can be seen in Figure 3.1A, the expected reduction in GSIS is not observed. We reasoned that this might be due to oxidation of the palmitate stock. To test this, the same BSA:palmitate conjugation protocol was executed with fresh palmitate stocks from various vendors (Figure 3.1B,C,D). INS1 cells cultured in 11mM glucose were similarly treated with palmitate from several sources at the designated respective concentrations in Figure 3.1B,C,D. These data do not show a significant

inhibition of GSIS at any of the concentrations used. There was a trend, albeit insignificant, in the BSA control, to reduce GSIS as compared to the INS1 control. A titration of palmitate was performed to show that a low dose of palmitate may be necessary to overcome the effect of FA-free BSA control on insulin secretion, since the controls are reduced to 1%FBS with addition of 100uM FA-free BSA. This could be due to the fact that BSA acts as a sink for lipid, since free fatty acids undergoing diffusion through the plasma membrane will be trapped outside the cell upon interacting with BSA (Hamilton, 1999).

We next sought to determine the relative contributions of palmitate and glucose to impairment of insulin secretion. INS1 cells cultured in 11mM glucose were treated either with 20mM glucose (GT), 0.4mM palmitate in 11mM glucose (LT) or a combination of 20mM glucose + 0.4mM palmitate (GLT). As can be seen in Figure 3.2A, the addition of palmitate (LT) increases basal insulin secretion, while addition of glucose (GLT) caused a further increase. However, the data indicate that the primary insult on GSIS is glucotoxicity. These experiments were performed in 1%FBS. Since INS1 cells are routinely cultured in 10% FBS, we hypothesized that serum reduction would sensitize beta cells to glucotoxic conditions. Hence, a dose response of various glucose concentrations was carried out with various serum concentrations (10% or 1%FBS). INS1 cells were co-cultured in the respective glucose concentrations indicated in Figure 3.2B,C at either 10% or 1%FBS. The results of these insulin secretion assays

indicated an increased sensitivity to the glucotoxic insult at lower serum concentrations. Moreover, glucotoxicity reduces GSIS in a dose-dependent manner.

Since fatty acids are typically complexed to BSA, this necessitates that the respective controls be treated with similar concentrations of FA-free BSA. Interestingly, we have found that overnight culture of INS1 cells in FA-free BSA results in a reduction of beta cell capacity to secrete insulin upon glucose stimulation in a dose dependent manner. Figure 3.3A,B demonstrate a reduction of GSIS in INS1 cells upon overnight exposure to the designated concentrations of BSA.

BSA has been shown to stimulate insulin secretion in acute conditions (Haber et al., 2003). In contrast, our data revealed a reduction of insulin secretion upon treatment with BSA in a dose dependent manner. These findings prompted us to examine the changes in intracellular lipid with various models of lipotoxicity. Erion et al recently demonstrated a left shifted response, or increased sensitivity of beta cells when cultured at high glucose concentrations or in excess lipid. These findings correlated with intracellular lipid content(Erion et al., 2015). To determine if changes in intracellular lipid could confound our results, we performed a comparison of our lipotoxicity protocol with that of Erion et al. INS1 cells grown in 11mM glucose were treated in either 10%FBS (normal culture

conditions), 50μM BSA 1%FBS, 100μM BSA 1% FBS, 100μM Oleate 10%FBS, or 400μM Palmitate 1%FBS. After 16h treatment with the respective nutrients, cells were fixed and stained with Bodipy493 and Hoescht to quantify lipid droplets and cell nuclei respectively. A high throughput imager (Operetta), with fixed filter sets, Bodipy493 Ex/Em 460-490, 500-550 and Hoescht Ex/Em 360-400, 410-480 was used to acquire images and quantify lipid droplets. We observed striking differences in lipid content among these conditions (Fig 3.4). The highest intracellular lipid content was observed with 100μM oleate in 10% FBS, while 100μM BSA in 1%FBS showed a drastic reduction in lipid content and number of lipid droplets per cell. Our presumed lipotoxic condition of 0.4mM palmitate in 1% FBS displayed a lower lipid content relative to normal 10% FBS culturing conditions. These data indicated that reduction of FBS to 1%, while warranted to increase BSA bound lipid of interest delivery to the cell, reveal a stark contrast to normal culture conditions, which may lead to misinterpretation of findings. It is important to emphasize that when a comparison to normal 10%FBS conditions is made, the reduction in insulin secretion is not significant, while the effect on intracellular lipid is substantial.

Having exposed intracellular lipid content as an important parameter influencing beta cell function, we resolved to develop a more suitable pathophysiological excess nutrient environment under which beta cell dysfunction may be probed. Beta cells have been shown previously to undergo

ER stress crystallization, since they are less equipped to handle saturated fatty acids due to lack of desaturase expression (Busch et al., 2005; Karaskov et al., 2006; Thörn et al., 2010). As such, it is more reasonable to utilize oleate, a lipid moiety the beta cell is equipped to handle. *In vivo* saturated fatty acids are never the sole fatty acid available to a cell. Given that beta cells are physiologically exposed to lower glucose concentrations than the normally accepted *in vitro* culturing conditions of 11mM glucose, we chronically cultured beta cells at 4mM glucose. INS1 cells were then treated at either 100μM or 150μM oleate at 4mM glucose for lipotoxicity or at similar concentrations of oleate while increasing glucose to 11mM as a glucotoxicity model. These studies revealed that 150uM oleate at 4mM glucose almost doubled basal insulin secretion while reducing GSIS (Fig.3.5A, B). Additionally, these data do not display a difference in insulin content as compared to the controls. Utilization of oleate directly complexed to BSA present in FBS as a lipotoxicity model may serve as a suitable model for the early changes in beta cell dysfunction during diabetes development, in which insulin is hyper-secreted at fasting/basal glucose levels, prior to manifestation of other long-term impairments.

Discussion

In vitro models of excess nutrients are valuable tools to investigate pathophysiological effects on cellular function. There is no clear consensus in the literature or guidelines for what an appropriate lipotoxicity model is. Additionally, the protocols employed to deliver fatty acids to cells are variable. Given that our palmitate model of lipotoxicity was challenging to reproduce, we attempted to understand the difficulties encountered. A key limitation of our system is the singular utilization of palmitate devoid of an unsaturated fatty acid in combination. We sought modifications that are experimentally reproducible and physiologically sound.

Technical Considerations

Developing an *in vitro* model of lipotoxicity is technically challenging due to the various elements involved. Batch to batch variability in fatty acids preparations affects reproducibility of data. We have determined that the source of palmitate may interfere with results, for reasons such as purity or oxidation of the samples. Since free fatty acids must be complexed to BSA to increase solubility and delivery to the cell, care must also be taken as to the source of BSA used. It is imperative that FA-free BSA is used. Furthermore, the method of purification is also important to avoid other contaminants, which may elicit an

inflammatory response, and thereby mask the influence of fatty acids, while also confounding interpretation of results. The temperature at which fatty acids are complexed is yet another critical parameter, since certain above threshold high temperatures may lead to aggregation of fatty acids. In our experiments, palmitic acid was dissolved in DMSO. Hence, it's also crucial to make certain that the palmitate is well dissolved in this organic solvent to ensure equimolar delivery of lipid to bind BSA during preparation of different lots.

Glucotoxicity is a Prerequisite for Detrimental Effects of Excess Lipids

Our findings indicate that 16h chronic incubation with glucose is the primary contributor to decreased beta cell function and reduction in GSIS in models of GLT. This is not a surprising result, since it has previously been shown(J. Fu et al., 2017) Glucose processing entails two energy-demanding processes. While metabolism of glucose is an overall energy generating process, ATP is required for the secretion of insulin. Importantly, ATP is required in the first committed step of glycolysis in which glucose phosphorylation is required to generate G-6-P. ATP is again required in the second committed step at PFK in conversion of fructose 6-phosphate to fructose 1,6-bisphosphate. Excess glucose demands ATP for glucose to be phosphorylated by hexokinase and PFK, therefore increasing energy demand. Cells chronically cultured in 11mM glucose vs. 4mM glucose have increased lipids. Glucose increases intracellular lipid by

providing a glycerol backbone for triglyceride synthesis. This in turn may result in futile TG/FFA cycling (Nolan, Madiraju, Delghingaro-Augusto, Peyot, & Prentki, 2006b).

Serum is Protective from Glucotoxicity

My data show that culturing INS1 cells in reduced serum sensitizes them to glucotoxicity. This suggests the presence of protective lipid moieties or factors that are lost upon serum reduction. In the case of higher serum, more glucose may be shuttled away from mitochondrial oxidative metabolism towards lipid esterification and formation of TG, or diverted to glycerol release. A recent study has shown a linear relationship for glycerol release, and lipid synthesis (FFA release FFA content and TG content) with increasing concentrations of glucose as mechanisms for excess fuel detoxification in beta cells (Mugabo et al., 2017). *Ex vivo* treatment of beta cells with serum from calorie-restricted animals vs. *ad libitum* fed animals (which is arguably more physiological) protects beta cells from glucolipotoxicity, by maintaining mitochondrial fusion capacity and respiratory function thereby indicating that factors present in the serum independent of nutrients influence adaptive responses to nutrient insults (Cerqueira et al., 2016). Alternatively, a potential protective factor in serum is GLP-1. Beta cells have been shown to secrete GLP-1 which contributes to their basal activity (Masur, Tibaduiza, Chen, Ligon, & Beinborn, 2005). GLP-1 may be supported by factors in the serum, and displays anti-apoptotic effects and is

protective from glucotoxicity and lipotoxicity (Buteau et al., 2004) (Cornu & Thorens, 2009). Discerning the effects of FAs vs. other factors in the serum, however, would necessitate treatment with equimolar concentrations of FA perhaps via utilization of delipidated serum.

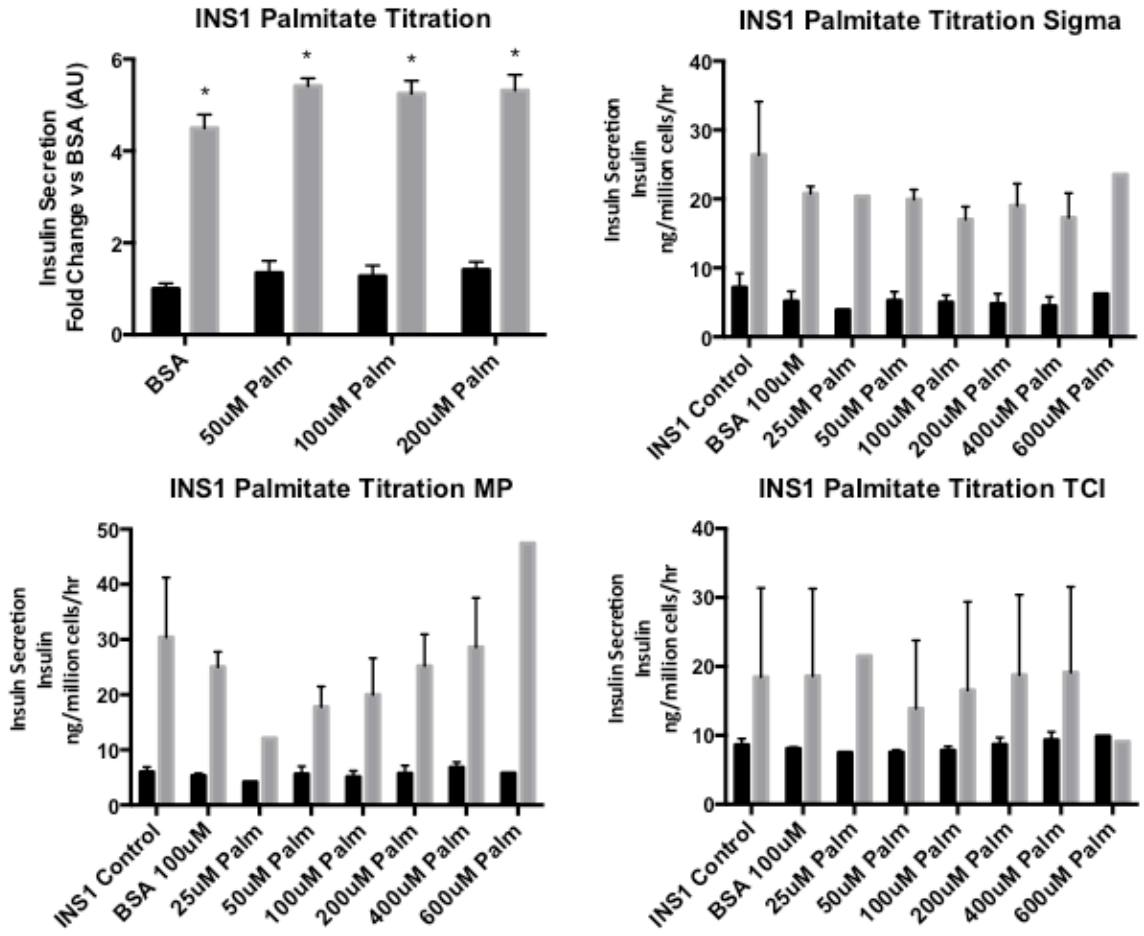


Figure 3.1. Optimization of Palmitate Lipotoxicity Model.

(A) INS1 cells cultured in 11mM glucose media were treated under concentrations of palmitate indicated for 16-20h, after which an insulin secretion assay was performed. n=3 *compared to BSA 2mM glucose $p<0.0001$ (B), (C), (D) n=2.

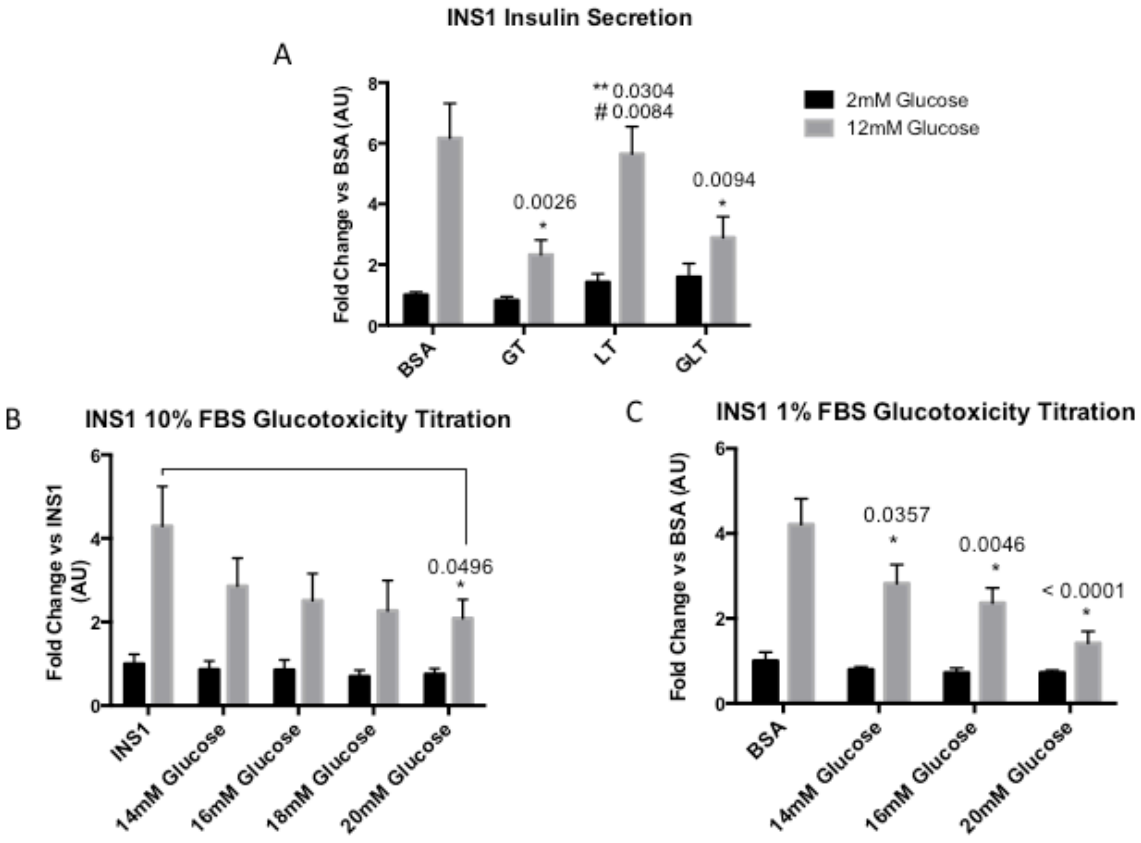


Figure 3.2. Comparison of Different Models of Excess Nutrients on beta cell Insulin Secretion.

INS1 cells cultured in 11mM glucose media were treated under different nutrient conditions for 16-20h, after which an insulin secretion assay was performed. (A) Glucotoxicity (GT) 20mM glucose, Lipotoxicity (LT) 0.4mM palmitate, 11mM glucose, Glucolipotoxicity (GLT) 0.4mM palmitate, 20mM glucose, n=3 * compared to BSA 12mM glucose, **compared to GLT 12mM glucose, # compared to GT 12mM glucose. Respective p values indicated above each condition. (B),(C). INS1 cells cultured in 11mM glucose were treated under glucose concentrations as indicated in either 1% or 10% FBS, n=3

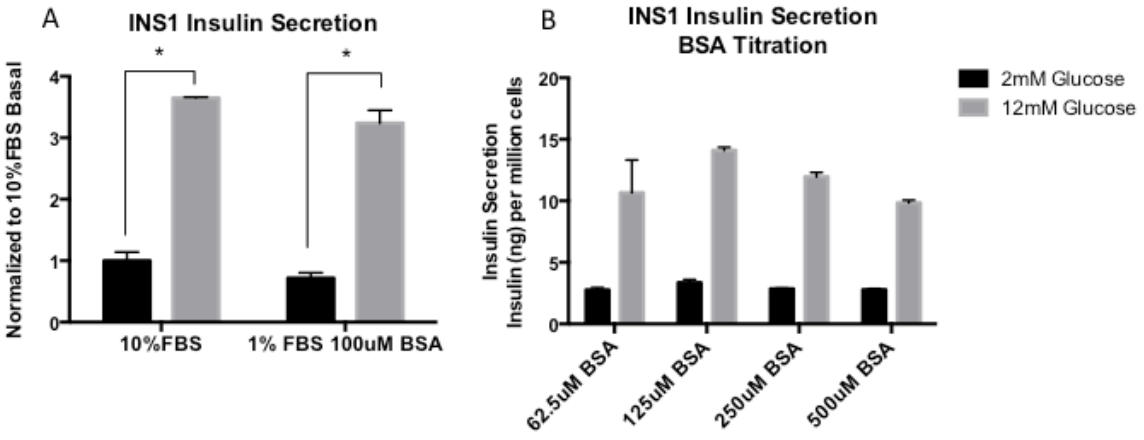
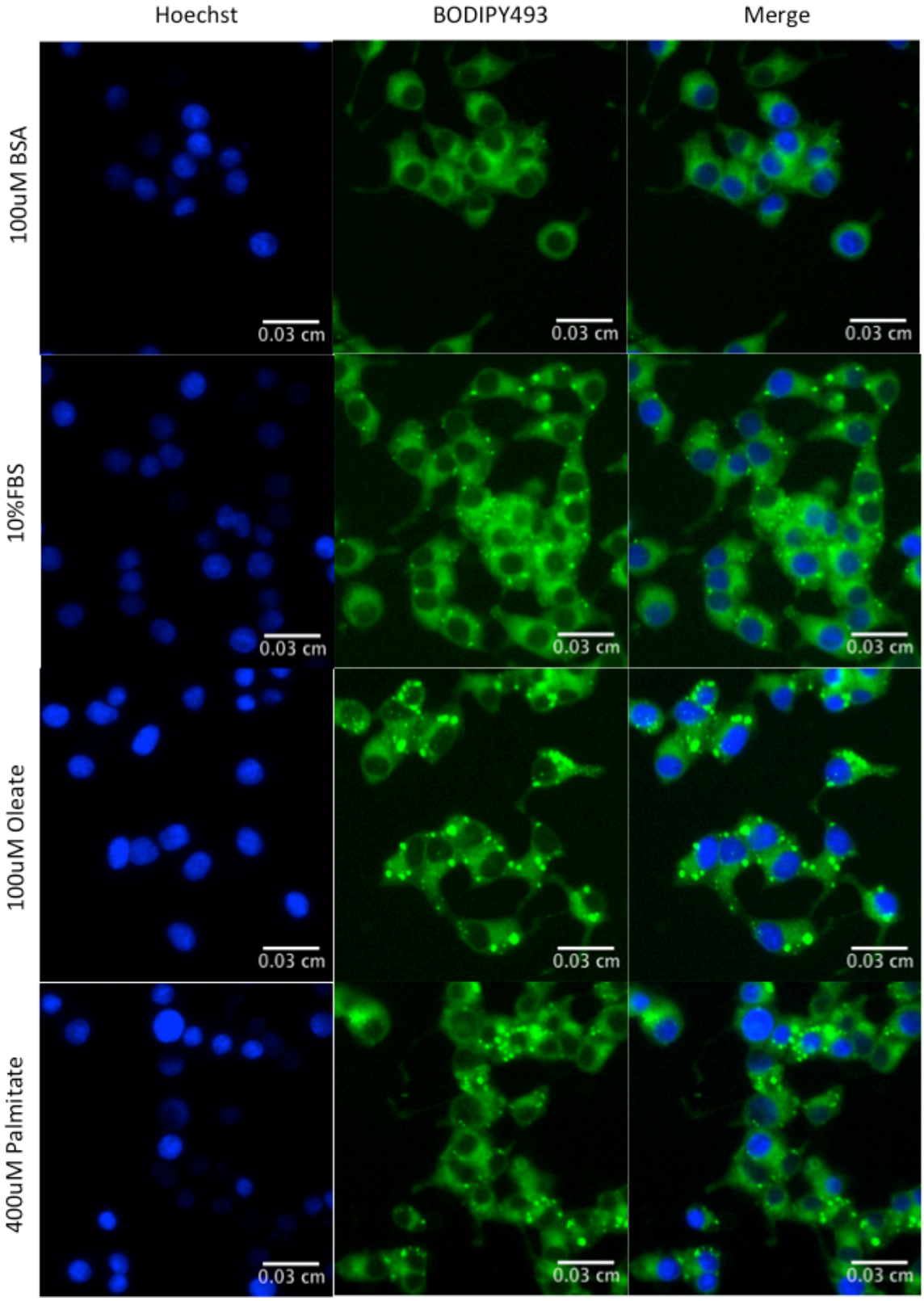


Figure 3.3. Influence of Serum reduction or addition of FA-free BSA on insulin secretion

(A) INS1 cells cultured in 11mM glucose were plated in 96-well plates and grown to 70% confluence. Cells were treated with either 10%FBS RPMI1640 INS1 Media or 1%FBS RPMI + 100uM FA-free BSA for 16-20h. n=3, *$p<0.0001$ (B) INS1 cells cultured in 11mM glucose. Titration of FA-free BSA performed at concentrations indicated in 1%FBS media for 16-20h. n=2. For insulin secretion assay, cells were preincubated in 2mM RPMI for 2h after which secretion assay was performed in Krebs as described in methods.


Hoechst
BODIPY493
Merge
100uM BSA
10%FBS
100uM Oleate
400uM Palmitate
0.03 cm

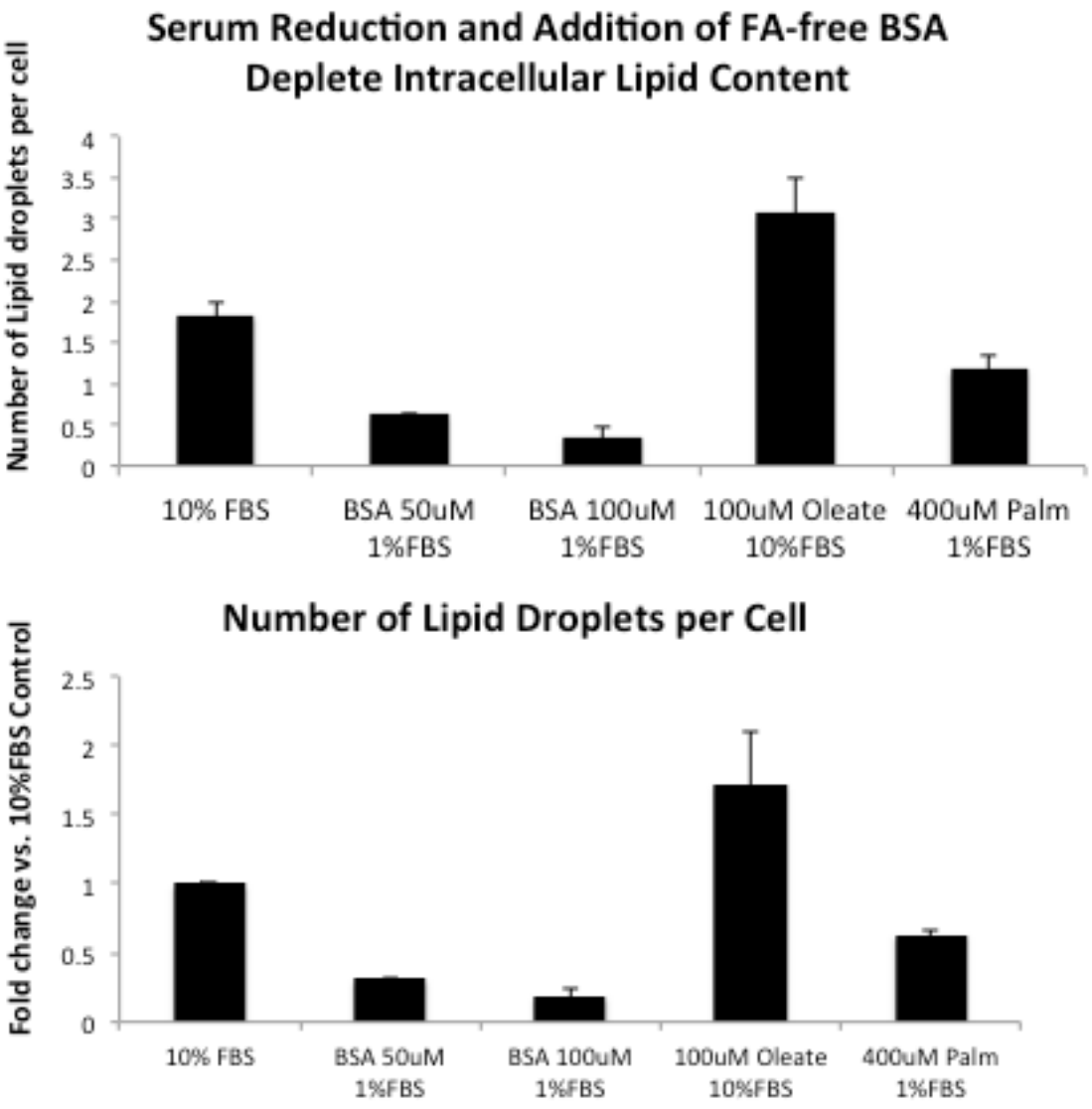


Figure 3.4 Reduction of Serum Depletes Intracellular Lipid

INS1 cells were grown to 70% confluence in 10%FBS RPMI1640 culturing media, after which they were treated with 1%FBS 50uM BSA, 100uM BSA or 400uM Palm conjugated to BSA in a 4:1 ratio. 100uM Oleate was conjugated directly to FBS.

Treatment time was 16h. Cells were fixed with 4%PFA for 15min and stained with Hoechst and BODIPY493 for 15min and subsequently washed twice with PBS and imaged using the Operetta imager. Bodipy493 Excitation 460-490, Emission 500-550, Hoescht Ex360-400 Em410-480.

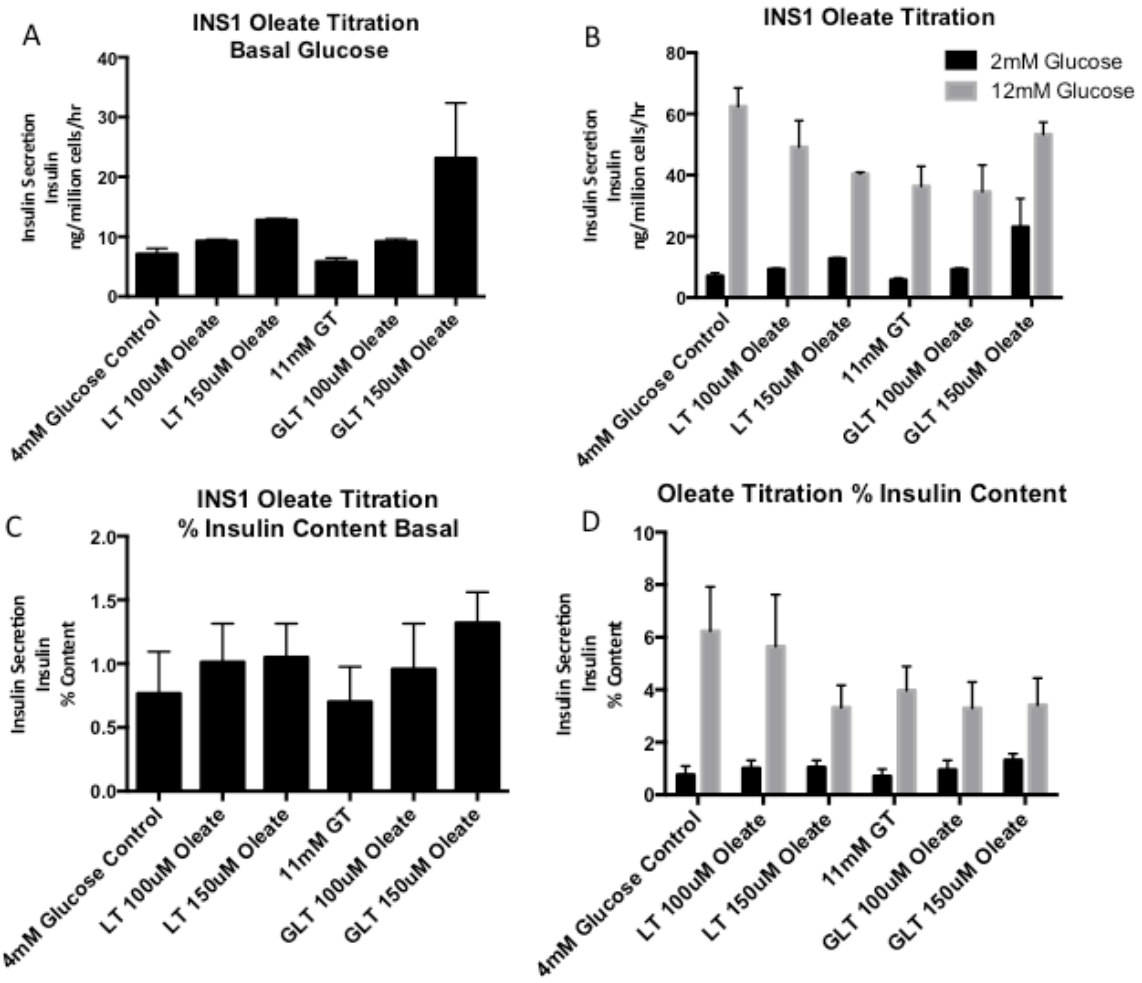


Figure 3.5. Oleate GLT Model-Lipotoxicity Increases Basal Insulin Secretion and Impairs GSIS

INS1 cells were cultured in 4mM glucose RPMI for at least 2 weeks. Cells were plated in 96-well plates and grown to 70% confluence. Titration of Oleate complexed directly to BSA in FBS was performed for a treatment time of 16-20h. LT= Lipotoxicity (100uM or 150uM Oleate, 4mM glucose). GT=Glucotoxicity, 11mM glucose. GLT=Glucolipotoxicity (100uM or 150uM Oleate, 11mM glucose). For insulin secretion assay, cells were pre-incubated in 2mM RPMI for 2h after which

secretion assay was performed in Krebs as described in methods. A-D, n=3. A, B, data normalized to ng of insulin per million cells per hour. C, D same data normalized as percentage of insulin content.

CHAPTER FOUR: Cyclophilin D-dependent Mitochondrial Proton Leak Regulates Insulin Secretion at Basal Glucose

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Introduction

Mitochondria are essential to pancreatic beta cell function (Maechler & Wollheim, 2001), as they are fuel integrators and generators of multiple signals such as ATP, ROS, GTP, and NADPH for insulin secretion (Wiederkehr & Wollheim, 2012) (Prentki, Matschinsky, & Madiraju, 2013). Beta-cell mitochondria are characterized by an inherently high level of bioenergetic inefficiency, since they display a high level of proton leak as compared to other cell types (Wikstrom et al., 2012). Leak represents the fraction of oxygen consumption that does not result in ATP production, but generates heat by dissipation of the proton motive force. Leak may occur in the basal state, and is a function of mitochondrial membrane potential and ATP/ADP ratio(Nicholls, 2016). Thus, under conditions of nutrient excess, the proportion of leak is augmented since metabolism of nutrients increases membrane potential and the ATP/ADP ratio (Liesa & Shirihai, 2013; Nicholls, 2016). These observations were first shown in isolated mitochondria derived from the clonal rat insulinoma cell line INS1 by the Brand lab(Affourtit & Brand, 2006), and subsequently confirmed by our group in primary mouse and human islets (Wikstrom et al., 2012).

Uncoupling reduces the bioenergetic efficiency of ATP production, which may contribute to the impairment of GSIS. Uncoupling protein 2 (UCP2) was proposed to mediate the beta cell proton leak. Initial data from whole body mixed background UCP2 KO mice revealed improved insulin secretory capacity,

improved glucose tolerance, and higher ATP and ROS levels(Joseph et al., 2004; S. C. Lee, Robson-Doucette, & Wheeler, 2009; C.-Y. Zhang et al., 2001). However, this was contradictory to data from UCP2 KO mice in a congenic background, where a normoglycemic phenotype with reduced insulin secretion and higher ROS production was observed (Pi et al., 2009). Convincing evidence opposing UCP2 as the source of the high leak in beta cells emerged from a beta cell specific KO model. $UCP2^{-/-}$ islets displayed no difference in oligomycin-insensitive respiration (leak) as compared to controls, even though a higher basal oxygen consumption rate (OCR) was observed. These islets have a higher ROS-dependent GSIS, with no effect on islet ATP content. While these mice are glucose intolerant, this was attributed to changes in alpha cell mass and dysregulation of glucose induced glucagon secretion as a result of increased islet ROS levels. Furthermore, this observation of reduced GSIS and increased oxidative stress was confirmed in 3 independent congenic UCP2 KO mouse strains (Pi et al., 2009) (Pi & Collins, 2010). As such, the mechanisms contributing to beta cell leak and how leak regulates beta cell insulin secretion is currently unknown.

Islets exposed to excess nutrients display a higher mitochondrial proton leak (Carlsson et al., 1999), in addition to higher basal insulin secretion(Fex et al., 2007). We have previously suggested that acute induction of mitochondrial leak mediates production of signals for insulin secretion, while chronic exposure to excess nutrients may be detrimental to mitochondrial health and beta cell

function(Liesa & Shirihai, 2013). Herein, we explored the relationship between leak and insulin secretion. There are multiple factors that may contribute to proton leak such as pores, electrogenic pumps, channels, or exchangers. Previous studies have revealed a signaling role of ROS in mediating basal insulin secretion (Pi et al., 2007; Saadeh et al., 2012). An attractive target is the PTP, since evidence suggests a low-conductance physiological role for the PTP, and that the PTP has been shown to be modulated by ROS and fatty acids (Bernardi et al., 2015).We hypothesize that leak induces insulin secretion via nutrient regulation of ROS-induced opening of the PTP and that this may contribute to hyperinsulinemia of the pre-diabetic state.

Results

Mitochondrial Proton Leak Stimulates Basal Insulin Secretion in Islets

Given the characteristically distinct and inherently high levels of proton leak in pancreatic beta cells, we sought to measure how proton leak may change in islets acutely exposed to various nutrients. To accomplish this, we employed our previously established high throughput method of intact islet respirometry to measure mitochondrial proton leak, which is the fraction of respiration that is independent of ATP-synthesis in cells with coupled mitochondria(Wikstrom et al., 2012). To establish the resting energy demand, oxygen consumption was

measured in human islets in the presence of 3mM glucose (basal). The same islet preparations were subsequently exposed either to 3mM glucose, 20mM glucose (stimulatory), or 3mM glucose with 10mM Leucine/Glutamine (5mM of each amino acid). Islets were then treated with the ATP synthase inhibitor oligomycin A to enable measurement of ATP independent respiration. This represents the fraction of oxygen consumed independently of generating ATP, thereby reflecting a dissipation of proton motive force. Despite a low concentration of glucose, the amino acids Leucine/Glutamine induced a similar amount of leak compared to stimulatory glucose levels (Figure 4.1A-B). To further understand how nutrient composition affects islet mitochondrial proton leak, mouse islets were exposed to either basal or stimulatory levels of glucose (3mM or 20mM respectively) with or without additions of 0.4mM palmitate or 10mM leucine/glutamine. Figure 4.1C, indicates that amino acids under basal glucose levels have the highest level of mitochondrial proton leak, irrespective of the glucose concentration, since the leak observed is similar to addition of amino acids under stimulatory levels of glucose. We next sought to determine if nutrient regulation of proton leak correlated with nutrient stimulation of insulin secretion. Indeed, amino acids enhanced insulin secretion under both basal and stimulatory glucose levels. This led us to hypothesize that mitochondrial proton leak may promote/mediate insulin secretion. Remarkably, islets treated with the uncoupler dinitrophenol (DNP) displayed increased proton leak and increased insulin secretion under basal/low glucose levels (Supplementary Figure 4.1 A and B).

However, DNP may have an effect on insulin secretion independent of mitochondrial uncoupling via depolarization of the plasma membrane. Therefore, an uncoupler, BAM15, which does not depolarize the plasma membrane (Kenwood et al., 2014) , was utilized to confirm this observation is a result of mitochondrial uncoupling. Indeed, Figures 4.1E and 4.1F show the increased proton leak and increased insulin secretion of mouse islets upon exposure to 1uM BAM15 in basal glucose.

Reactive Oxygen species Increase Mitochondrial Proton Leak and Basal Insulin Secretion in Islets

While chronic excess ROS/oxidative stress are known to have damaging effects on beta cell function (Oprescu et al., 2007), ROS have also been implicated as a signal for insulin secretion (Pi et al., 2007; Saadeh et al., 2012). Since we have established that mitochondrial proton leak stimulates insulin secretion, our next objective was to elucidate the relationship between ROS and proton leak. We first wanted to determine changes in ROS production upon nutrient treatment. Since the combination of the amino acids Leucine/Glutamine promoted the largest increase in leak (Figure 4.1 C), these nutrients were selected to determine changes in ROS. We hypothesized that nutrient-induced ROS production mediates leak and insulin secretion. The fluorescent probe, DCFDA, which assesses reactive oxygen and nitrogen species (Kalyanaraman et

al., 2012), was utilized to measure differences in the redox state of mouse islets treated with basal 3mM glucose or 10mM Leu/Gln in addition to 3mM glucose with or without the antioxidant MnTBAP. Figure 4.2A indicates an increase in ROS upon incubation with amino acids that is abrogated upon co-incubation of MnTBAP. This result corresponded with a decrease of mitochondrial proton leak as seen in Figure 4.2B. This suggests ROS are necessary to promote nutrient induced changes in proton leak. To further strengthen these findings, we performed a gain of function experiment. Incubation of islets in hydrogen peroxide induced insulin secretion at basal glucose levels (Fig 4.2C). To determine the effect of increased ROS on leak, the pro-oxidant, menadione was employed to determine if ROS would induce leak as seen in Fig 4.2D. Menadione consumes NADPH via redox cycling, thereby reducing the antioxidant ability of glutathione (Criddle et al., 2006). Addition of the antioxidant MnTBAP did not significantly change BAM15-stimulated insulin secretion (Fig 4.2E), suggesting that ROS is upstream of proton leak.

Amino-acid and Fatty Acid Stimulation of Insulin Secretion at Basal Glucose is ROS-dependent.

Our data suggest that ROS stimulate insulin secretion and proton leak under basal glucose, and that ROS is upstream of leak. Given that amino acid induced leak is ROS dependent, we hypothesized that amino acid induced

insulin secretion at low (basal) glucose is contingent upon an increase in ROS. To test this, mouse islets were incubated in basal glucose with addition of amino acids in the presence or absence of MnTBAP. MnTBAP significantly decreased the amino acid effect on insulin secretion. This confirms that the effect of acute treatment of amino acid induced insulin secretion is ROS-dependent. Furthermore, to determine if the increase in ROS is necessary for other nutrient induced insulin secretion under basal glucose levels, we tested the effect of a combination of saturated and unsaturated fatty acids, oleate/palmitate. While fatty acids were shown to increase leak (Fig 4.1C) and insulin secretion (Fig 4.1D), we observed a trend toward a decrease in basal secretion in the presence of oleate/palmitate upon treatment with the antioxidant EUK (Fig 4.3B). EUK reduction of basal insulin secretion with oleate/palmitate was only performed in two independent experiments. This result perhaps suggests separate mechanisms by which amino acids and fatty acids induce basal insulin secretion (under low/basal glucose conditions), since treatment with antioxidants had a much greater response in insulin secretion stimulated by amino acids.

The Mitochondrial Permeability Transition Pore Regulates Proton Leak and Insulin Secretion in Islets

We have established that mitochondrial leak is sufficient to induce insulin secretion and that ROS mediates this effect of leak on secretion in a nutrient

dependent manner. The key outstanding question was to determine the source of the leak. It has previously been established that the mitochondrial permeability transition pore can also function in a physiological low conductance state that is separate from its high-conductance state that initiates cell death (Ichas & Mazat, 1998; Yehuda-Shnaidman, Kalderon, Azazmeh, & Bar-Tana, 2010). Indeed, the PTP can be activated by ROS or by FFA (Bernardi et al., 2015). While the overall structure and components of the mPTP are still debated in the field, a key established regulator of the PTP is Cylophilin D, a peptidyl prolyl cis-trans isomerase located in the mitochondrial matrix (Giorgio et al., 2017). The absence of CypD increases the threshold required of calcium to induce PTP opening, maintaining the PTP in a closed state (Basso et al., 2005). Cyclophilin D expression is shown to increase with aging (Moreno-Asso, Castaño, Grilli, Novials, & Servitja, 2013) and evidence indicates an increase of Cyclophilin D expression in diabetic islets as compared to non-diabetic controls (Taneera et al., 2012) (Taneera et al., 2013) (Kanatsuna et al., 2013). Hence, we hypothesized that PTP could be the source of the leak. To determine if the PTP contributes to the leak, NIM811, a cyclosporine derivative that is an inhibitor of cyclophilins, was used (Waldmeier, Feldtrauer, Qian, & Lemasters, 2002). NIM811 is an alternative to CsA, which avoids the off-target effects of inhibiting calcineurin and therefore does not possess immunosuppressive effects. Pharmacological inhibition of CypD with Nim811 significantly reduced amino acid (Leucine/Glutamine)-induced proton leak and insulin secretion (Fig 4.3A and Fig

4.3B). To test if this phenomenon was nutrient specific, we determined if NIM811 had a similar effect on fatty acids, since they are known to induce PTP opening. To do so, a combination of oleate and palmitate, complexed to BSA in FBS, was used to stimulate insulin secretion and leak with or without addition of the PTP inhibitor NIM811. NIM811 also prevented the FA induced increase on insulin secretion and leak (Fig 4.3C and Fig 4.3D).

Role of Cyclophilin D on Leak, Basal and Glucose Stimulated Insulin Secretion

Given that pharmacological approaches have a higher risk of off-target effects, a genetic approach was applied to determine the effects of cyclophilin D ablation in beta cell leak and secretion. Deletion of CypD is a validated model for inhibition of PTP opening, as it necessitates a higher calcium threshold to activate the PTP (Basso et al., 2005). Islets were isolated from whole body Cyclophilin D KO C57BL6J mice at 12 weeks. The profile of these islets was characterized ex vivo under basal and stimulatory glucose levels. KO islets and corresponding WT littermate control islets were incubated in either basal 3mM glucose, or stimulatory 20mM glucose to measure leak or insulin secretion in independent experiments. CypD deficient islets displayed lowered proton leak revealed by oligomycin insensitive respiration, and insulin secretion under low glucose conditions, while no significant difference was observed under stimulatory levels (GSIS). (Fig. 4.5A, 4.5B and Supplementary Fig 4.2A,B).

The mitochondrial permeability transition pore regulates proton leak and insulin secretion in islets from high fat-fed mice

Exposure of islets to excess nutrients impairs beta cell function in a time dependent manner (Paolisso, Gambardella, et al., 1995a; Sako & Grill, 1990). Since it has been shown that pre-diabetic islets from obese mice have increased gene expression of CypD and increased basal secretion (Taneera et al., 2012) (Taneera et al., 2013) (Kanatsuna et al., 2013), we hypothesized that islets from high fat diet (HFD) fed mice would reveal higher protein expression of CypD. Fig 4.6A and B, show that islets obtained from mice fed a HFD (60% fat, 12weeks) have higher expression of CypD. Indeed, these islets display basal insulin secretion and proton leak that is sensitive to pharmacological inhibition of CypD by NIM811 (Fig.4.6 C,D), suggesting that increased CypD dependent leak may be contributing to basal hyperinsulinemia.

Proton leak induced insulin secretion requires generation of mitochondrial GTP

It is intriguing that mild mitochondrial uncoupling amplifies basal and nutrient stimulated insulin secretion. This suggests that leak enables generation of a signal that may amplify insulin secretion. Mild uncoupling will likely lead to increased TCA cycle flux to generate the same amount of ATP. In previous studies, the Kibbey lab has shown that mitochondrial GTP generation is a signal

modulating insulin secretion (Kibbey et al., 2007). This observation came from comparison of the roles of two isoforms of the TCA cycle enzyme, Succinyl-CoA Synthetase. While both isoforms metabolize the same substrate, one produces GTP (SCS-GTP) while the other produces ATP (SCS-ATP) as a byproduct. The mitochondrial GTP isoform has been shown to amplify insulin secretion via increased PEP cycling and serves as an indicator for TCA cycle flux (Fig. 4.7A, Supplementary Fig. 4.3). Mitochondrial PEPCK utilizes mGTP to convert oxaloacetate to PEP, which is exported from the mitochondria and converted to pyruvate via pyruvate kinase (PK) (Stark & Kibbey, 2014; Stark et al., 2009). It is mechanistically unclear how mitochondrial derived PEP amplifies insulin secretion (Stark & Kibbey, 2014). We hypothesized leak increases TCA cycle flux and concomitantly increases mGTP production. To answer this question, INS1 cells expressing the ATP isoform of succinyl CoA synthetase (hSCS-ATP) at a much higher ratio when compared to SCS-GTP (inhibition of SCS-GTP by competition), or control cells, which express equally both the ATP and GTP isoforms of SCS were treated with BAM15 under basal 2mM glucose conditions. BAM15 significantly stimulated insulin secretion in the control INS1 cells as compared to the hSCS-ATP cells, indicating that proton leak stimulated insulin secretion requires the generation of mitochondrial GTP (Fig 4.7B). To determine if this is also the mechanism by which leak regulated insulin secretion in an acute or chronic excess nutrient condition, INS1 cells expressing either the ATP or GTP isoforms of SCS were acutely treated with the amino acids Leu/Gln (45 min)

or with 150uM oleate (48h), after which insulin secretion was assayed. Figure 4.7C indicates that amino acids acutely increase basal insulin secretion (glucose 2mM) in INS1 cells in a manner that requires generation of mitochondrial GTP. Figure 4.7D indicates that chronic oleate treatment does not increase basal insulin in INS1 expressing hSCS-ATP isoform, while there is a 2-fold increase in the hSCS-GTP isoform. Thus, this result suggests that SCS-GTP activity might be required for excess fatty acids to increase basal secretion.

Discussion

While the majority of beta cell research focuses on mechanisms of stimulating GSIS, our study reveals a novel bioenergetic mechanism that regulates basal insulin secretion (under low glucose). These findings provide an innovative means by which a potential therapeutic target for hyperinsulinemia of the pre-diabetic state may be developed. This mechanism promotes insulin secretion under low glucose conditions upon both acute and chronic exposure to excess nutrients. We have shown that increased mitochondrial leak acutely increases insulin secretion, while chronic exposure of islets to excess nutrients results in leak that leads to decreased bioenergetic efficiency. While previous studies have proposed that leak may be contributed by UCP2 or the ANT, no convincing evidence has been demonstrated in beta cells (Nicholls, 2016). Our investigation on the role of mitochondrial proton leak on beta cell function has revealed that leak stimulation of insulin secretion is mediated by the PTP and

that this pathway is dependent upon ROS or physiological mixtures of fatty acids oleate and palmitate.

We have shown that acute treatment of islets with fatty acids stimulates basal insulin secretion and increased mitochondrial leak. We also show that leak itself, via addition of a specific mitochondrial uncoupler, is sufficient to stimulate insulin secretion at basal glucose levels. This suggests that the increase in leak promotes the production of additional mitochondrial signals that amplify insulin secretion signaling. We determined that leak stimulates insulin secretion via increased GTP generation by the TCA cycle, as our experiments in which the different isoforms of SCS were manipulated to generate either ATP, GTP or both, revealed that leak induction of insulin secretion requires generation of mitochondrial GTP.

Previous studies have implicated a signaling role for ROS in insulin secretion (Pi et al., 2007; Saadeh et al., 2012). Here, we reveal that acute amino acid treatment increases insulin secretion in a ROS-dependent manner, as treatment with MnTBAP reduces the amino acid stimulated increases in insulin secretion. Most importantly, we identify the PTP as the source of the nutrient induced leak, as addition of the CypD inhibitor, NIM811 reduces Leu/Gln and Oleate/Palmitate insulin secretion and leak.

Our working model places ROS upstream of the leak for amino acids, since addition of MnTBAP did not reduce BAM15 mediated insulin secretion.

Further studies may be needed to differentiate ROS scavenging or uncoupling for non-specific drug effects. It is noteworthy to mention that amino acids potently induce secretion as compared to fatty acids, while they maintain similar increases on proton leak. This may be due to the dose of nutrients utilized or other effects of the amino acids leucine and glutamine. In addition to being metabolized and activating TCA cycle activity, the increased secretion may result from direct effects of leucine/amino acid-derived metabolites on the plasma membrane K_{ATP} channel (Newsholme, Bender, Kiely, & Brennan, 2007). Furthermore, EUK did not appear to have a strong inhibitory effect on fatty acid induced insulin secretion. This suggests the possibility that the mechanism by which leak is induced is nutrient dependent. Whereby amino acids may increase leak via increased TCA cycle flux and consequent ROS generation, fatty acids may induce leak by directly activating the PTP in an acute setting. However, in a chronic setting, increased generation of mitochondrial GTP is required for fatty acids to increase basal insulin secretion.

We have shown that increased leak observed in islets from HFD animals is sensitive to NIM811, supporting the hypothesis that increased CypD expression in diabetic islets (Taneera et al., 2012) (Taneera et al., 2013) (Kanatsuna et al., 2013) may be contributing to increased basal insulin secretion and hyperinsulinemia. Future studies will be necessary to test whether beta cell

specific ablation of CypD is protective under HFD, as this would enable identification of a therapeutic target to prevent hyperinsulinemia in the pre-diabetic state.

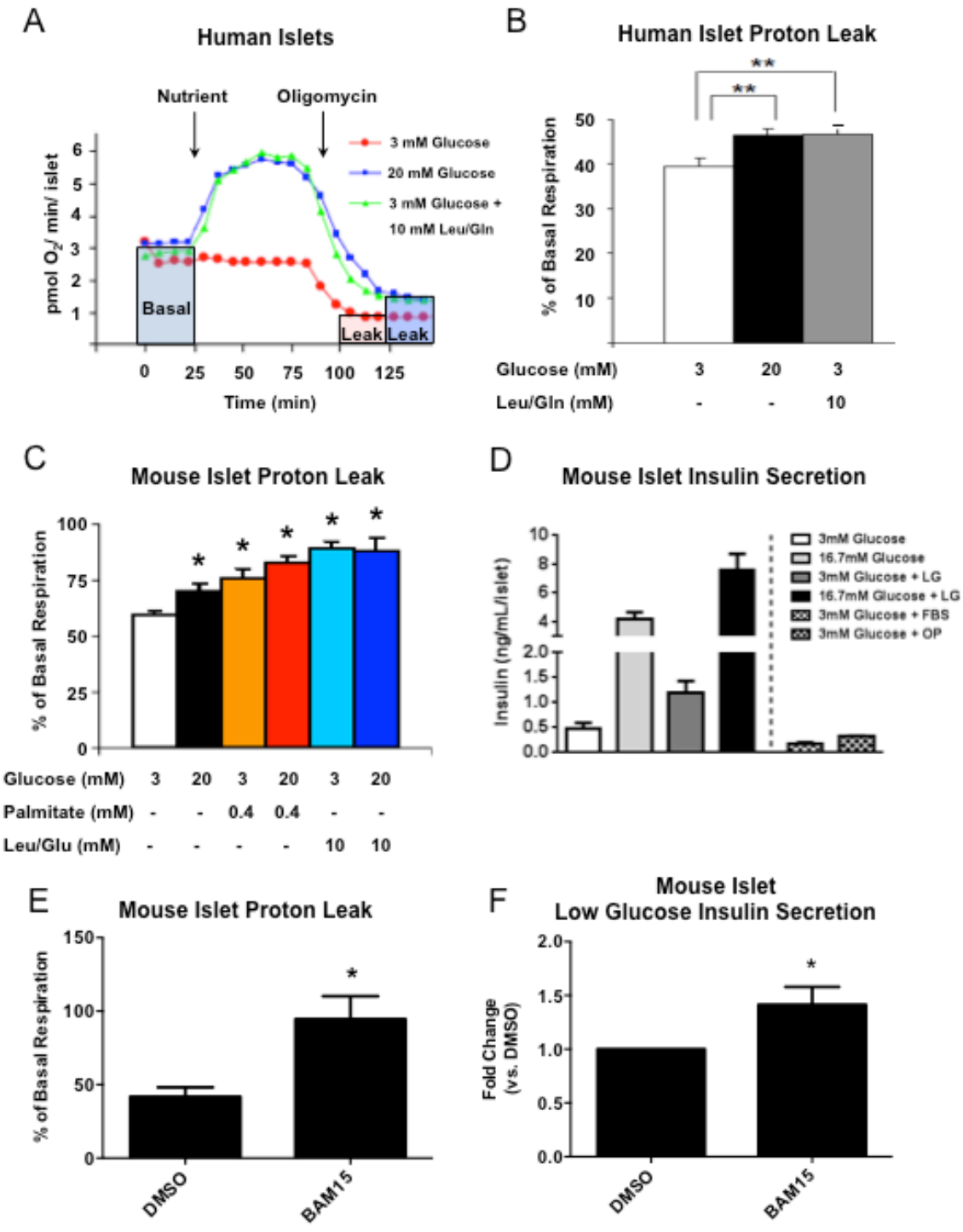

A
Human Islets
Nutrient
Oligomycin
3 mM Glucose
20 mM Glucose
3 mM Glucose +
10 mM Leu/Gln
pmol O2/ min/ islet
Basal
Leak
Leak
Time (min)
B
Human Islet Proton Leak
% of Basal Respiration
Glucose (mM) 3 20 3
Leu/Gln (mM) - - 10
C
Mouse Islet Proton Leak
% of Basal Respiration
Glucose (mM) 3 20 3 20 3 20
Palmitate (mM) - - 0.4 0.4 - -
Leu/Glu (mM) - - - - 10 10
D
Mouse Islet Insulin Secretion
Insulin (ng/mL/islet)
3mM Glucose
16.7mM Glucose
3mM Glucose + LG
16.7mM Glucose + LG
3mM Glucose + FBS
3mM Glucose + OP
E
Mouse Islet Proton Leak
% of Basal Respiration
DMSO
BAM15
F
Mouse Islet
Low Glucose Insulin Secretion
Fold Change
(vs. DMSO)
DMSO
BAM15

Figure 4.1. Mitochondrial Proton Leak Stimulates Basal Insulin Secretion in Islets.

(A) Representative traces showing absolute values of human islet oxygen consumption. Basal respiration was measured over time, and changes in respiration were assessed after sequential addition of different nutrients and the mitochondrial ATP synthase inhibitor oligomycin to calculate mitochondrial proton leak. Islets were incubated in 3mM glucose and then injected with media with 3mM glucose (red trace), 20mM glucose (blue trace) or 10mM leucine + glutamine (5mM of each amino acid, green trace). (B) Mitochondrial proton leak of human islets after addition of 3mM glucose, 20mM glucose or 10mM leucine + glutamine. Proton leak is expressed as the oligomycin insensitive respiration normalized as a percentage of basal respiration. n = 4-10 donors. (C) Mitochondrial proton leak from mouse islets exposed to either 3mM or 20mM glucose, in the absence or presence of 400μM palmitate conjugated to BSA or 10mM leucine + glutamine. n=4 independent experiments, each containing islets pooled from 8 mice fed a chow diet. (D) Insulin secretion from mouse islets incubated in either 3 or 16.7mM glucose in the absence or presence of 10mM leucine + glutamine or 90μM fatty acids (60μM oleate/30μM palmitate complexed to FBS) or FBS control. n = 6-10 independent experiments. (E) Mitochondrial proton leak in mouse islets treated with 1μM of the uncoupler BAM15. n = 3 independent experiments. (F) Basal insulin secretion in 3mM glucose

Krebs from BAM15-treated mouse islets. n = 5 independent experiments. All data are means ± standard error of the mean (SEM). For B, ** indicates $p<0.001$ obtained with Student t test when compared to 3 mM glucose injection. For C, E and F, * indicates $p<0.05$ in a Student t-test.

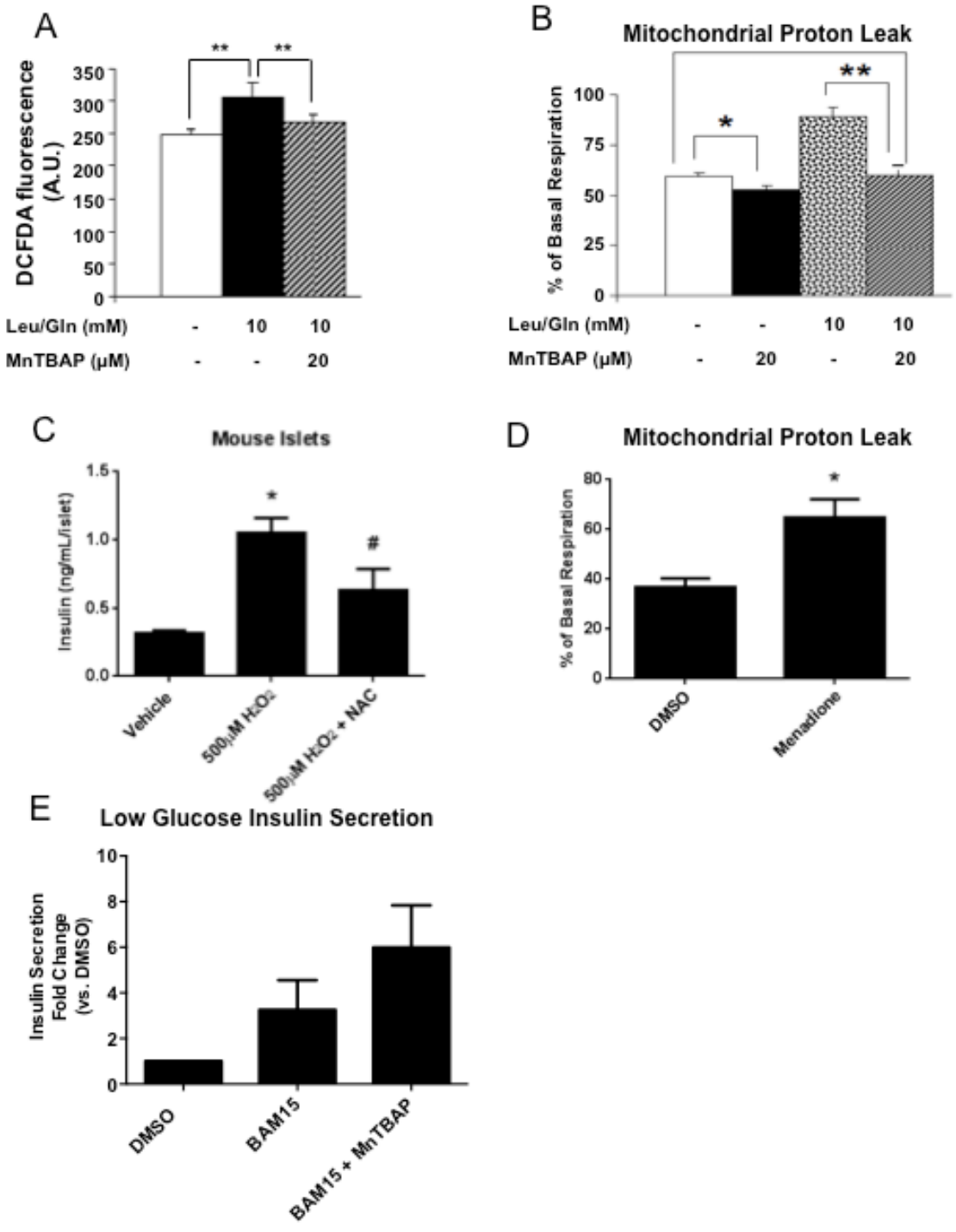


Figure 4.2. Reactive Oxygen Species Increase Mitochondrial Proton Leak and Basal Insulin Secretion in Islets.

(A) Reactive oxygen species (ROS) were measured in dispersed islet cells after staining with 10μM CM-H2DCFDA for one hour and incubation with 3 mM glucose in the absence or presence of 10mM leucine/glutamine and 20μM MnTBAP for 15min. n = 4 independent experiments. (B) Mitochondrial proton leak from islets acutely exposed to 3mM glucose or 10mM leucine/glutamine in the absence or presence of 20μM TBAP. n = 4 independent experiments.(C) Insulin secretion from islets incubated in 3mM glucose Krebs buffer, or 3mM Glucose Krebs buffer in the presence of 500uM H2O2 with or without addition of NAC. n= 3-5 independent experiments, $p<0.05$ compared to vehicle (*) or H2O2 (#) by one-way ANOVA. (D) Mitochondrial proton leak measured in islets treated with the ROS-generating compound menadione (10μM) or DMSO vehicle. Islets were incubated with menadione for 30 minutes prior the start of respirometry. n = 4 independent experiments. (E) Insulin secretion from islets incubated in 3mM glucose Krebs buffer in the absence or presence of 1μM BAM15 or 20μM TBAP. Islets were exposed to compounds for 45 min, during the secretion period. n = 7-8 independent experiments. All data are means ± SEM.. For A, ** indicates $p<0.001$ Student t-test. For B, * indicates $p<0.05$; ** indicates $p<0.001$ of Student t-test. For D, * indicates $p<0.05$ Student t-test.

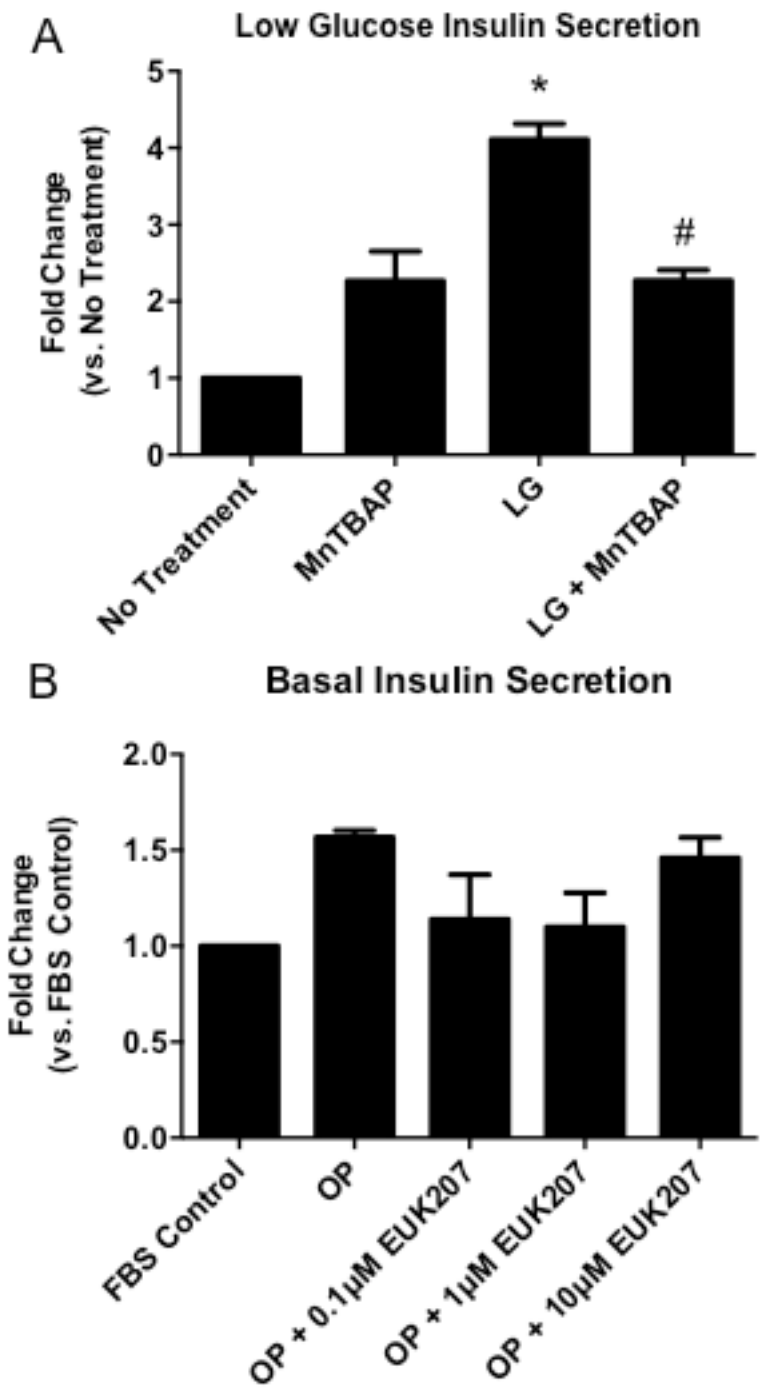


Figure 4.3. Amino-acid and Fatty Acid Stimulation of Insulin Secretion at Basal Glucose is ROS-dependent.

(A) Amino acid-induced insulin secretion in 3mM glucose from mouse islets treated with 20μM MnTBAP (10mM leucine/glutamine). n = 3-4 independent experiments, $p<0.05$ compared to vehicle (*) or Leu/Gln (#) by one-way ANOVA (B) Fatty acid-induced insulin secretion in 3mM glucose from mouse islets treated with EUK-207 (Oleate/Palmitate 150uM, 2:1 ratio, total 225uM). n=2

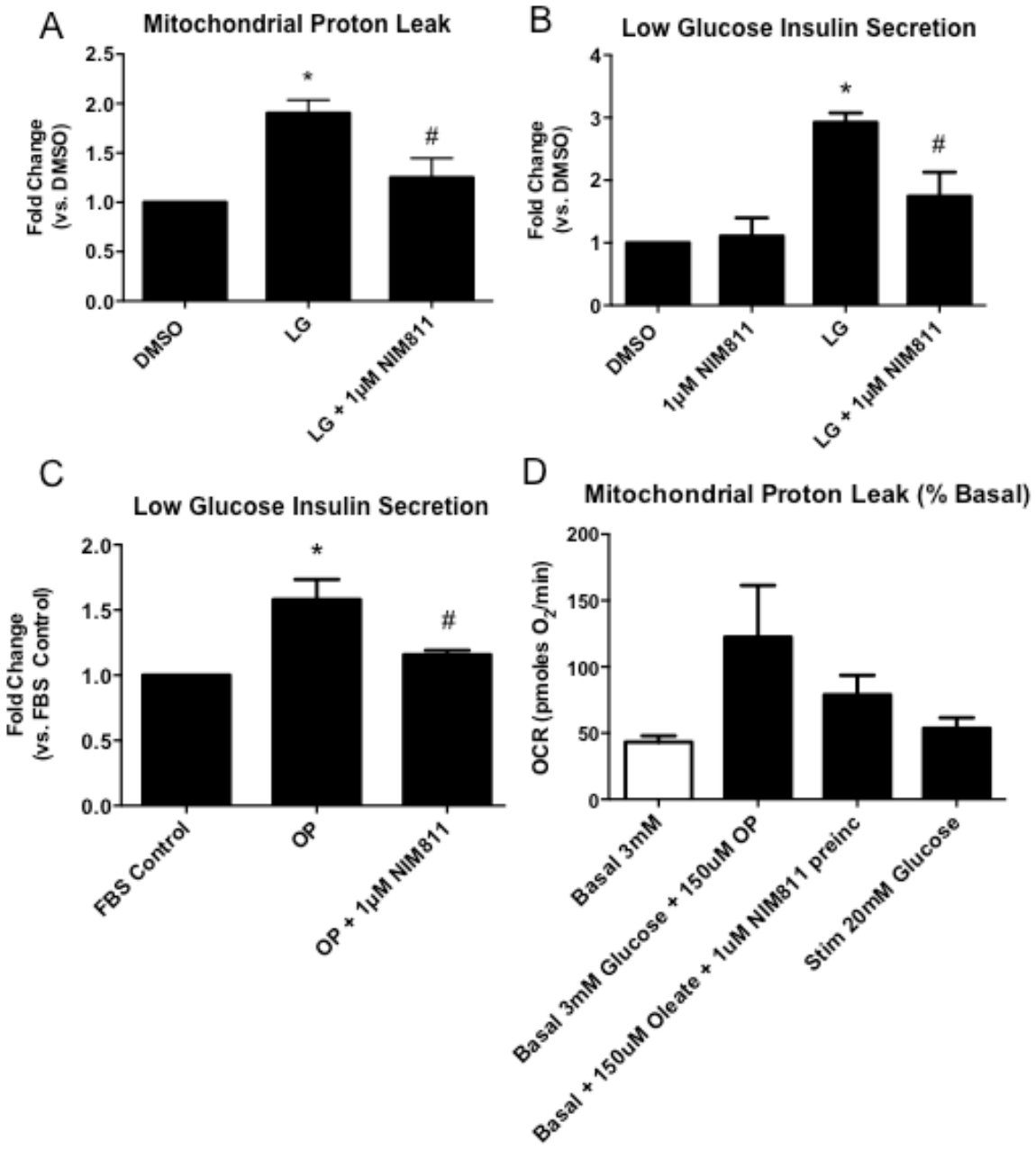


Figure 4.4. The Mitochondrial Permeability Transition Pore Regulates Proton Leak and Insulin Secretion in Islets.

(A) Mitochondrial proton leak in islets acutely exposed to 10mM leucine/glutamine (Leu/Gln) in the absence or presence of the cyclophilin D (CypD) inhibitor NIM811. Islets were treated with DMSO vehicle or 1μM NIM811 in Seahorse media containing 3mM glucose for 1hr prior to respirometry. n = 4 independent experiments. (B) Insulin secretion from islets incubated with 10mM LG in 3mM glucose in the absence or presence of 1μM NIM811. Islets were exposed to NIM811 during both the 30min pre-incubation and 45 min secretion periods, for a total of 75min. n = 4-5 independent experiments. (C) Insulin secretion from islets incubated with 225uM fatty acids (150uM oleate/75uM palmitate) conjugated to FBS or FBS control diluted in 3mM glucose Krebs buffer. Islets were incubated with the fatty acids for the 45min secretion period. n = 3 independent experiments. (D) Mitochondrial proton leak in islets acutely treated with 225uM fatty acids (150uM oleate/75uM palmitate). n=1. For A-B, significant difference compared to DMSO control (*) or 10mM LG (#) by one-way ANOVA. For C, significant difference compared to FBS control (*) or OP (#) by one-way ANOVA. OCR, Oxygen Consumption Rate.

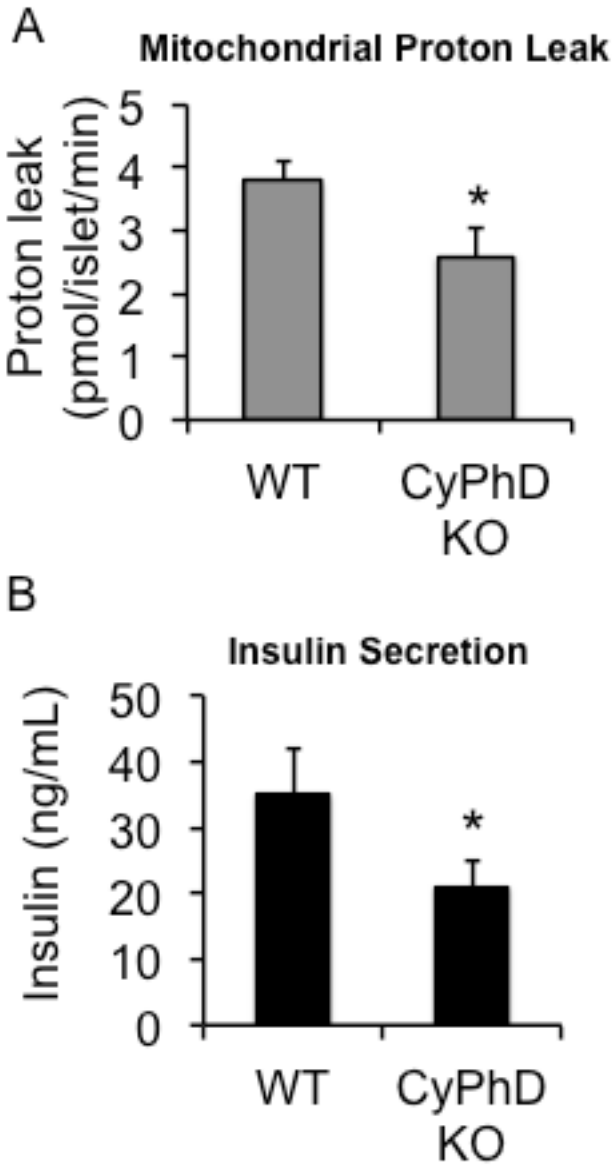


Figure 4.5. Cyclophilin D Deficient Islets Display Lower Mitochondrial Proton Leak and Insulin Secretion at Basal Glucose

(A) Basal insulin secretion in islets isolated from CypD KO mice; n=5 per genotype (B) Mitochondrial proton leak is reduced in islets from CypD KO mice; n=3 mice per group. Error bars represent ± SEM; *, $p<0.05$ by Student's *t* test.

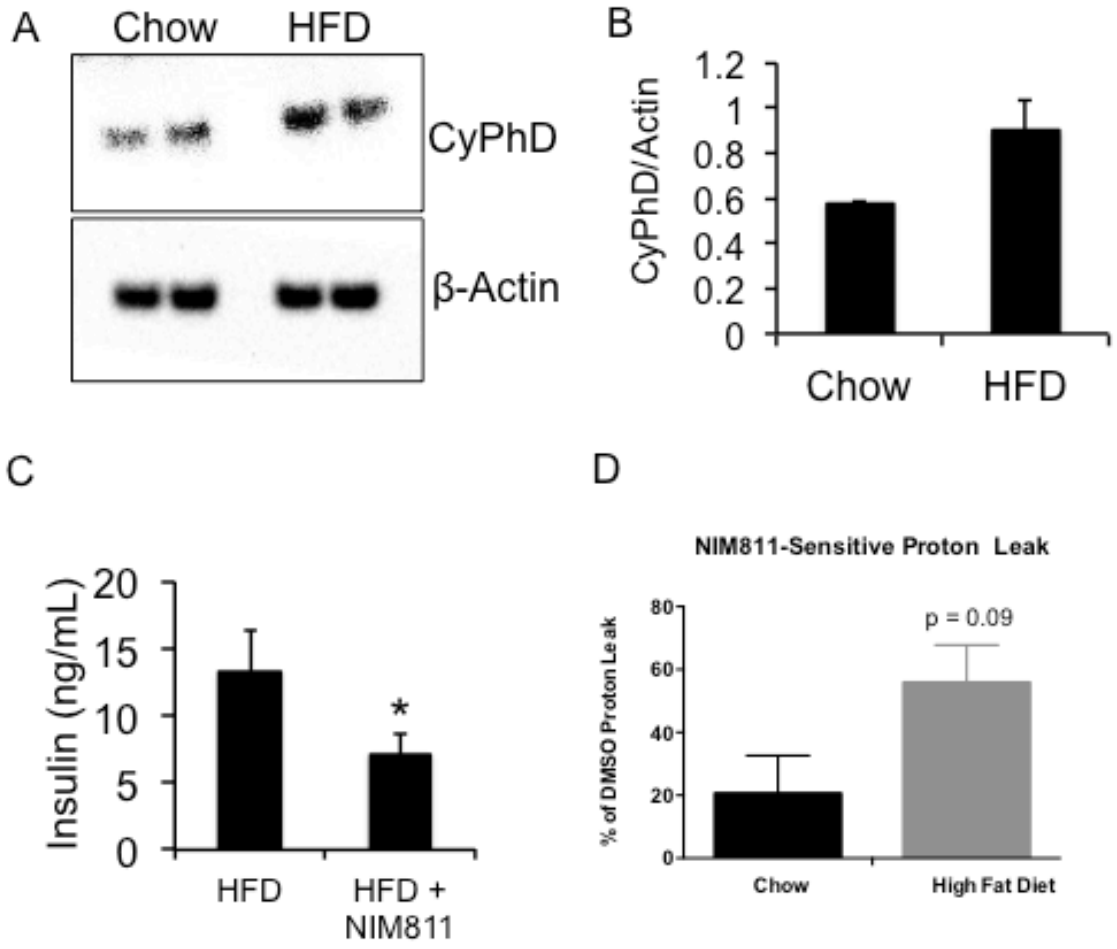


Fig 4.6. The Mitochondrial Permeability Transition Pore Regulates Proton Leak and Insulin Secretion in HFD Mouse Islets

(A) Western blot image and (B) quantification of 2 independent islet isolations from mice fed a chow or HFD (60% kcal from fat for 12 weeks); n=2 mice per group. (C) NIM811 reduces basal insulin secretion in 3mM glucose in islets isolated from HFD mice. n=3, Error bars represent ± SEM; *, $p<0.05$ by Student's *t* test. (D) NIM811-

sensitive proton leak in islets from chow- or HFD-fed mice. NIM811-sensitive leak was calculated by subtracting the leak in the presence NIM811 from the average respective DMSO control leak value, dividing this value by the DMSO control leak and multiplying by 100. n = 3 (Chow) or 4 (HFD) independent experiments. p = 0.08 by two-tailed, unpaired t-test.

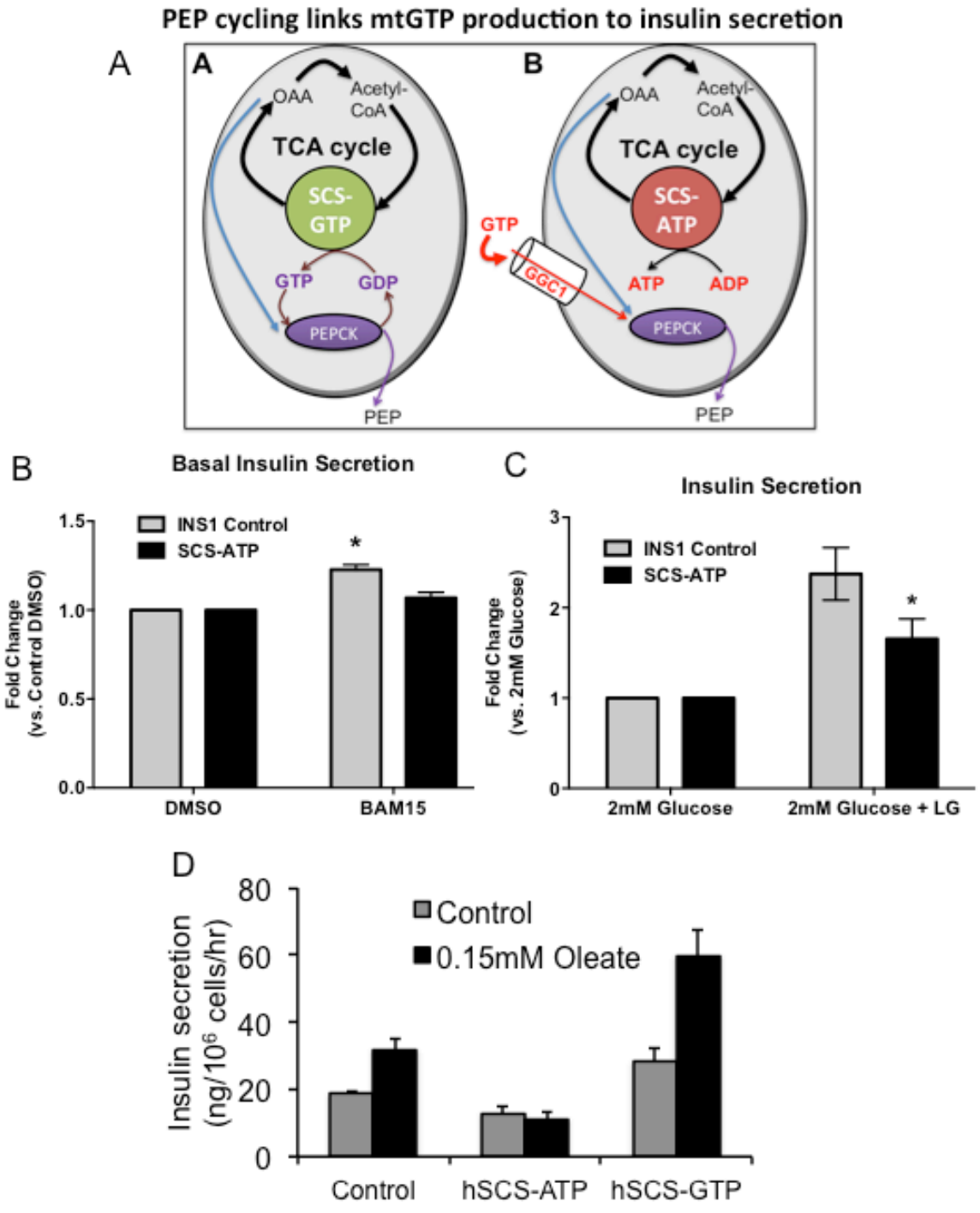
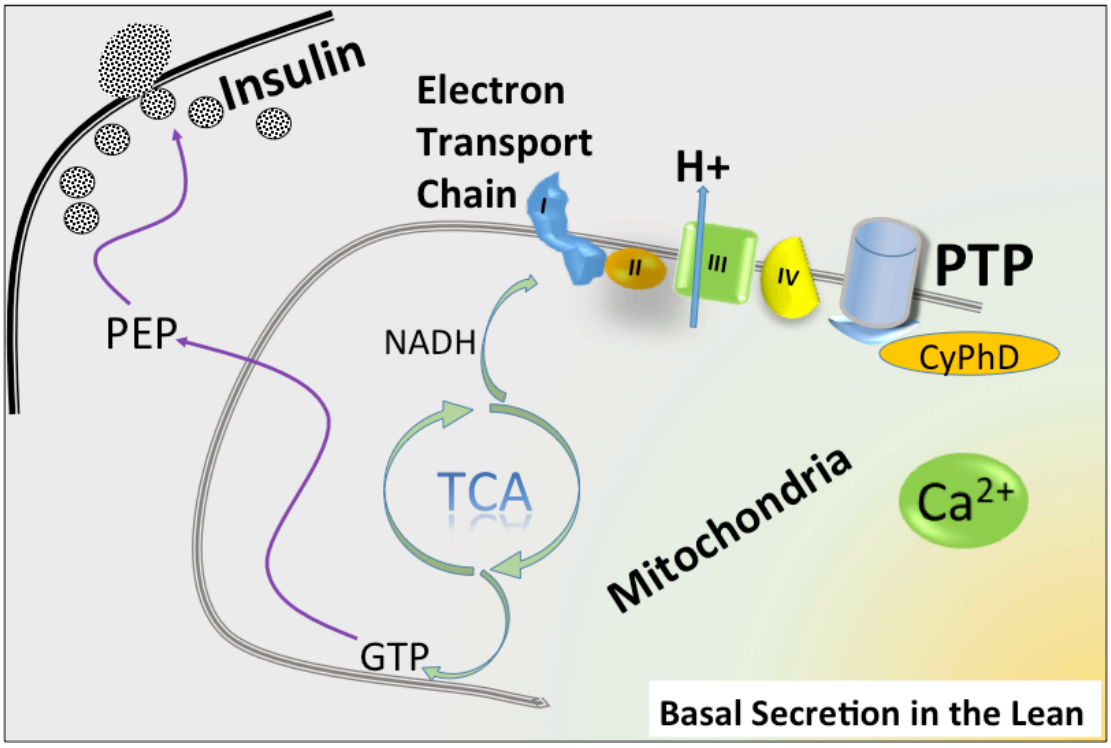

PEP cycling links mtGTP production to insulin secretion
A
A
OAA
Acetyl-CoA
TCA cycle
SCS-GTP
GTP
GDP
PEPCK
PEP
B
OAA
Acetyl-CoA
TCA cycle
SCS-ATP
GTP
GGC1
ATP
ADP
PEPCK
PEP
B
Basal Insulin Secretion
INS1 Control
SCS-ATP
Fold Change (vs. Control DMSO)
1.5
1.0
0.5
0.0
*
DMSO
BAM15
C
Insulin Secretion
INS1 Control
SCS-ATP
Fold Change (vs. 2mM Glucose)
3
2
1
0
*
2mM Glucose
2mM Glucose + LG
D
Insulin secretion (ng/10^6 cells/hr)
80
60
40
20
0
Control
0.15mM Oleate
Control
hSCS-ATP
hSCS-GTP

Figure 4.7. Proton leak induced insulin secretion requires generation of mitochondrial GTP

(A) Schematic displaying PEP cycling links mGTP production to insulin secretion. (Stark, Kibbey PMID:19635791) Since GTP cannot exit mitochondria, PEPCK converts oxaloacetate and GTP into PEP and GDP. GDP is recycled by SCS-GTP while PEP is exported from the mitochondria and has been shown to increase insulin secretion. The mechanism by which this occurs remains unclear. Increased production of PEP resulting from overexpression of the SCS-GTP isoform results in greater insulin secretion. The SCS-ATP isoform does not contribute to increased PEP production or export from mitochondria resulting in lower insulin secretion. (B) INS1 cells expressing hSCS-ATP have reduced basal insulin secretion in response to mitochondrial uncoupler BAM15. n=3, , $p<0.05$ by two way ANOVA compared to DMSO control. (C) Acute amino acid data in ATP/GTP cells. n=4 $p<0.05$ compared to INS1 control 2mM glucose + Leu/Gln by two way ANOVA. (D) Chronic Oleate treatment does not increase basal insulin secretion in INS1 cells expressing hSCS-ATP isoform. n=2


Insulin
Electron
Transport
Chain
H+
I
II
III
IV
PTP
CyPhD
PEP
NADH
TCA
Mitochondria
Ca2+
GTP
Basal Secretion in the Lean

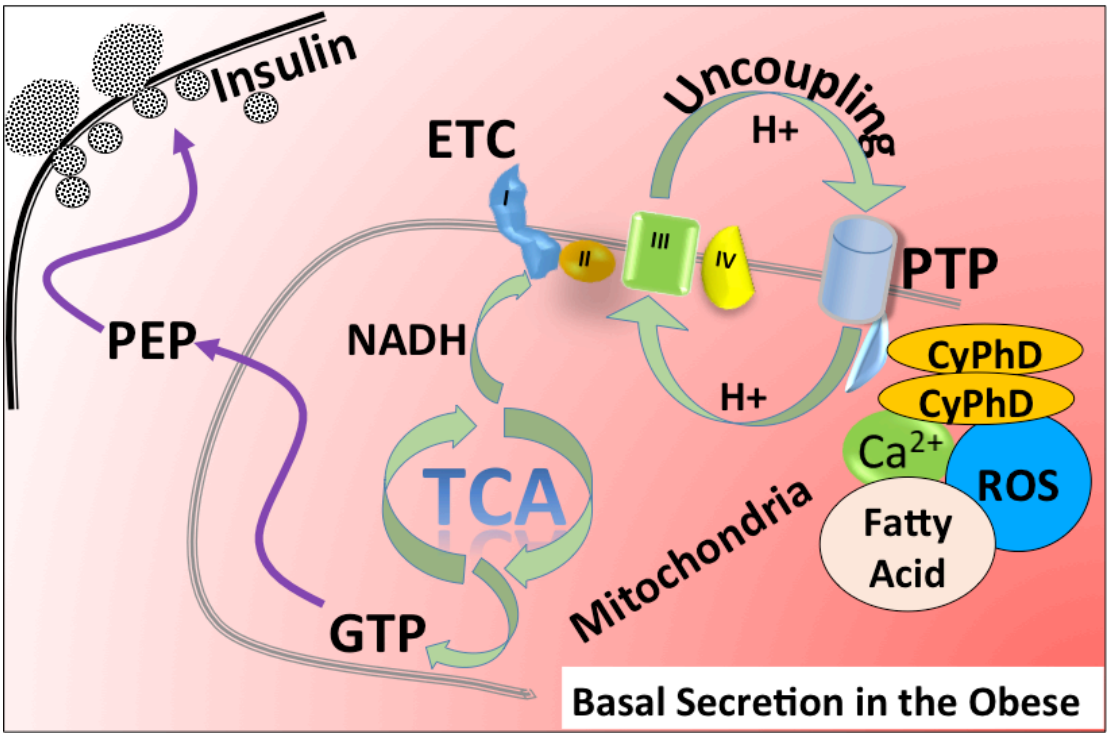
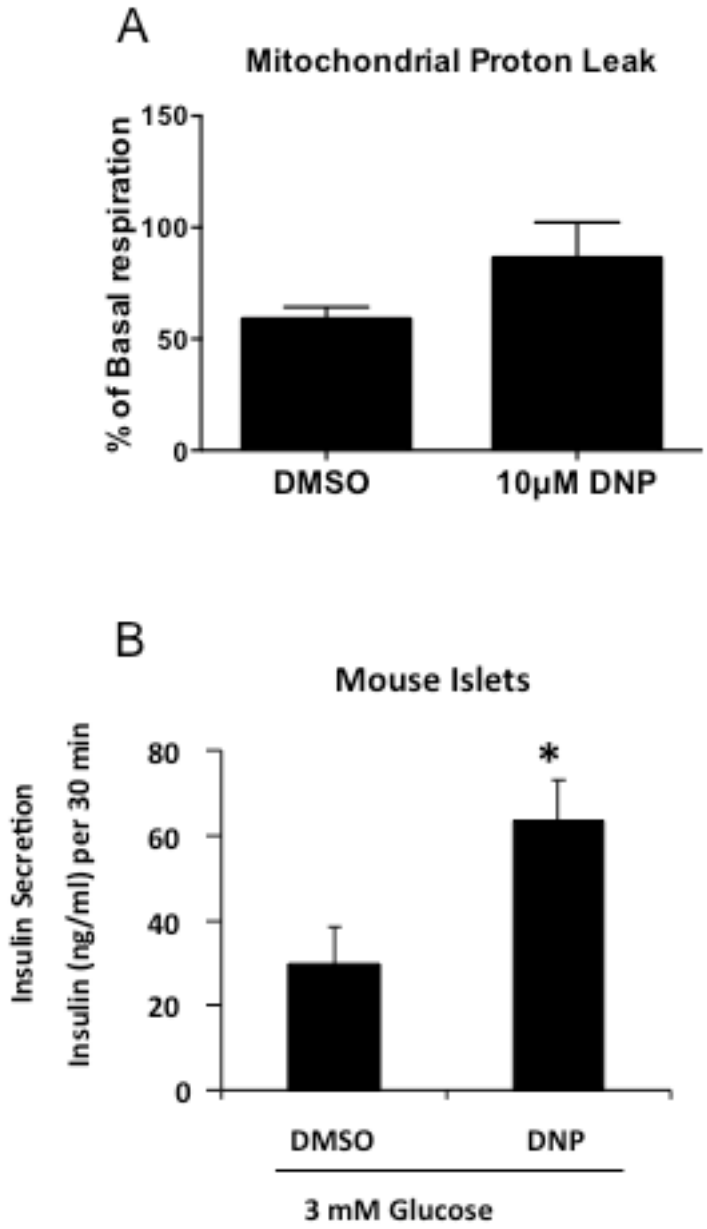

Insulin
ETC
Uncoupling
H+
I
II
III
IV
PTP
PEP
NADH
CyPhD
CyPhD
H+
Ca2+
ROS
TCA
Mitochondria
Fatty
Acid
GTP
Basal Secretion in the Obese

Figure 4.8. Mechanism of Elevated Insulin Secretion at Basal Glucose Concentrations.

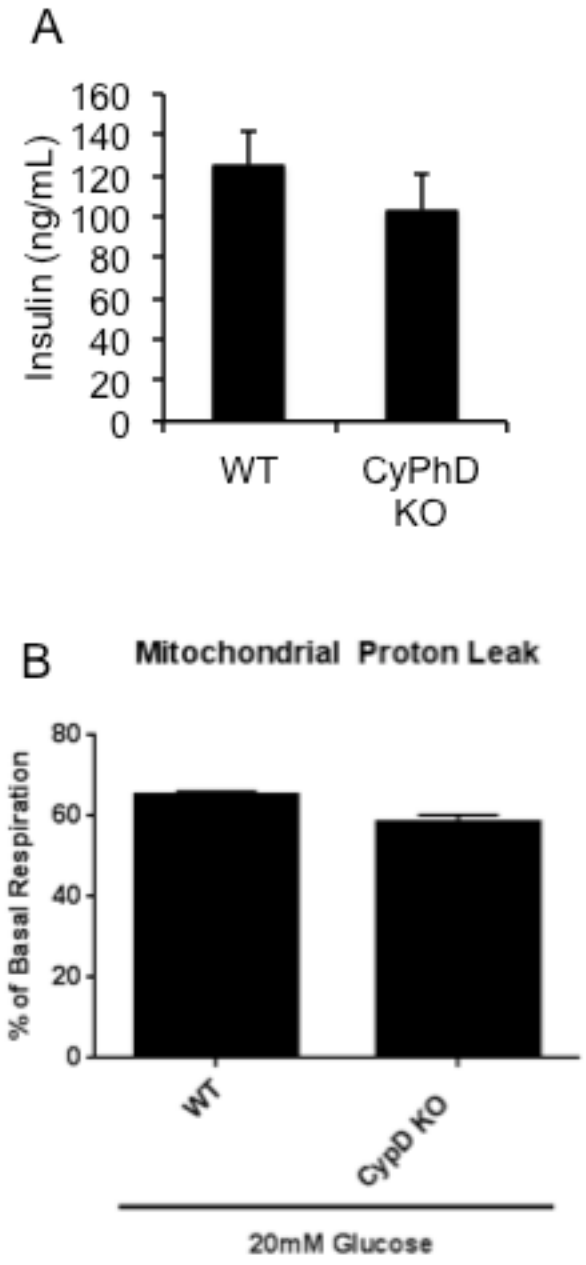
Increase in insulin secretion at basal glucose concentrations is the result of uncoupling mediated by a proton leak through the Permeability Transition Pore (PTP). In the obese state, the PTP becomes activated due to increased expression of the PTP activator, CyPhD. CyPhD increases the PTP sensitivity to Ca^{2+}, ROS and FAs. Proton leak through the PTP consumes NADH and increases the flux in the TCA cycle as the mitochondria attempt to maintain membrane potential, which in turns generates GTP. Mitochondrial GTP amplifies phosphoenolpyruvate production, which is a coupling factor for insulin secretion.



Supplementary Figure 4.1. Mitochondrial bioenergetics and insulin secretion in mouse islets treated with uncouplers.

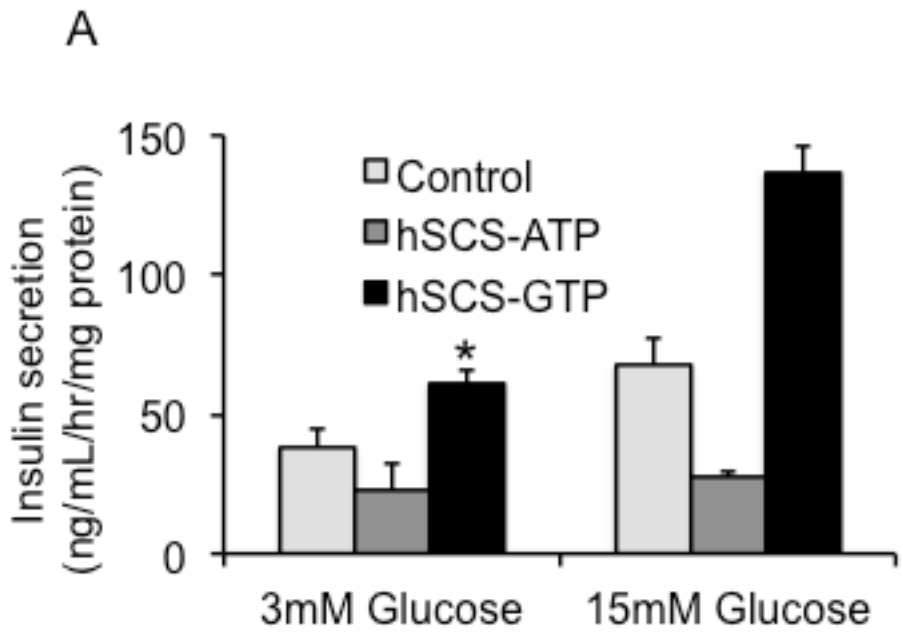
(A) Proton leak in mouse islets acutely exposed to 10μM dinitrophenol (DNP) or DMSO vehicle. n =1 independent experiment. (B) Insulin secretion from mouse islets

incubated with DNP or DMSO for 30min in 3mM glucose. n = 3 independent experiments. Data are means ± SEM. *p < 0.05 by unpaired, two-tailed t-test.



Supplementary Figure 4.2. Ablation of CypD does not reduce insulin secretion or leak under stimulatory glucose levels.

(A) GSIS in CyPhD KO islets is not significantly reduced; n=3. Error bars represent ± SEM; *, $p<0.05$ by Student's *t* test. (B) Mitochondrial proton leak under stimulatory glucose in CypD vs WT islets. n=2 mice per genotype.



Supplementary Figure 4.3. Mitchondrial GTP increases both basal and glucose stimulated insulin secretion.

(A) Transient overexpression of the ATP and GTP isoforms of SCS reduces and increases LGIS in INS1 cells, respectively; n=6 *p<0.05

CHAPTER FIVE: General Discussion

T2D is a multifactorial metabolic disease characterized by insulin resistance, impaired beta cell function, and hyperglycemia. The conventional model of T2D progression argues that insulin resistance of the peripheral tissues drives the hypersecretion of insulin from beta cells as a compensatory mechanism. This increased beta cell workload ultimately impairs beta cell capacity to secrete insulin due to beta cell exhaustion and eventual failure. However, an alternative theory provides compelling evidence supporting the hypothesis that hyperinsulinemia may be the primary defect which prompts changes in insulin sensitivity (Corkey, 2012b). In this case, the development of insulin resistance may be a compensatory response to avoid hypoglycemia, in addition to increased adiposity and negative effects of insulin signaling in other tissues (Erion & Corkey, 2017). Genome wide association studies indicate that majority of T2D risk genes reflect beta cell dysfunction (Florez, 2008). The only current treatments that restore beta cell function, secretory capacity, and response to glucose are fasting and bariatric surgery(Lim et al., 2011; Reed et al., 2011). Here, hyperinsulinemia is normalized independent of and prior to changes in insulin resistance. Furthermore, elimination of the beta cell insulin gene is protective from diet induced obesity (Mehran et al., 2012). Elucidating the cellular mechanisms that regulate basal insulin secretion and beta cell function, would allow us to test the role of hyperinsulinemia in the development of diabetes

and potentially identify therapeutic targets that may prevent or delay progression to T2D. In this thesis, I have developed an *in vitro* model of excess nutrients, and outline important parameters of glucolipotoxicity that must be considered. I have also identified a mechanism by which changes in beta cell bioenergetic function as it relates to PTP-mediated leak regulates beta cell insulin secretion in the basal state.

Glucolipotoxicty: Challenges, Considerations, and Implications for β-cell Function

In Chapter 3, I demonstrate the difficulties encountered when developing a protocol for a nutrient excess model. I also highlight the important factors that must be considered for technical reasons, as well as physiological implications for beta cell function. These findings resulted in a publication reviewing the problems encountered and solutions developed amongst our group and Prof Kowaltowski's group (Alsabeeh, Chausse, Kakimoto, Kowaltowski, & Shirihai, 2018). Excessive levels of circulating fatty acids observed in obesity are thought to deteriorate beta cell function, but exactly how fatty acids can change beta cell function is not well understood. Hence, *in vitro* development of glucolipotoxicity models allows for mechanistic insight on a cellular level. Fatty acids are insoluble in aqueous solutions, and are complexed to FA-free BSA, in a manner emulating the physiological or pathophysiological state.

The protocols by which fatty acids are complexed and applied for *in vitro* excess nutrient supplementation differ widely in the beta cell research field. A review of the literature displays variability of experimental design in the mode of FA supplementation regarding the percentage of serum in the media and addition of FA-free BSA in the controls. In some studies FA are dissolved in ethanol and directly added to culture media containing 10% FBS with an equivalent amount of ethanol to controls(Y. P. Zhou & Grill, 1994). Another study dissolves FA in DMSO, complexes to BSA, and added to media with 10%FBS, while the control media contains the equivalent FA-free BSA and DMSO(Maedler, Oberholzer, Bucher, Spinas, & Donath, 2003). Other studies reduce the percentage of FBS in the media (Las et al., 2011), while others are performed in serum free media (Karaskov et al., 2006). My results indicate that FA-free BSA causes a reduction in insulin secretion in a dose-dependent manner, while quantification of intracellular lipids shows reduction in lipid droplet content upon reduction of serum and addition of FA-free BSA. Although there is no empirical evidence to dissect the contribution of FBS reduction vs. addition of FA-free BSA, I speculate that the reduction in serum is likely to have a larger effect compared to addition of FA-free BSA in the controls. These factors are crucial to keep in mind, since FBS percentage in the media and/or addition of FA-free BSA in the controls may not only affect lipid content, but also likely sensitize the cells and influence other factors such as induction of autophagy in the case of a reduction in serum. The

mode of fatty acid preparation and administration must be considered to achieve robust experimental design.

The ratio of FA:BSA is often not reported in the literature. This is an extremely important and overlooked aspect, since the ratio at which FAs are complexed to BSA dictates the fraction of bound/unbound free fatty acids, and determines the concentration of fatty acid the cells will be exposed to. This may create difficulty in interpretation of data from different labs in addition to the aspect of reproducibility from different FA preps. To circumvent this, confirmation via measurement of total FFAs or unbound FFAs may be valuable (Alsabeeh et al., 2018).

β-cell Specific Physiological Considerations

Traditionally, the INS1 pancreatic beta cell line is cultured in media containing 11mM glucose. Erion et al have shown that culturing these cells in high glucose or excess lipids results in a left-shifted response to GSIS, indicating they are more sensitized for insulin release at lower glucose concentrations (Erion et al., 2015). This potentially explains the phenomenon of basal hyperinsulinemia in the pre-diabetic obese state. Culturing INS1 cells in 4mM glucose, may require a longer growth time, but would reflect a right-shifted, more physiological condition under which there is a reduced intracellular lipid content

as seen in non-pathological islets. In addition to careful consideration for normal glucose culture conditions, studies of beta cells employing models of lipotoxicity or glucolipotoxicity in which only saturated fatty acids such as palmitate are supplemented are not physiological. It is important to note that a mixture of circulating saturated and unsaturated fatty acids exists *in vivo*. Moreover, palmitate is toxic to beta cells, contributing to formation of crystals and ER stress response, apoptosis, and inflammatory response (Busch et al., 2005; Karaskov et al., 2006). Oleate is protective from the negative effects of palmitate. Hence, the use of oleate or a combination of oleate and palmitate may be physiologically more accurate.

Proton Leak Regulation of Insulin Secretion

While it has been known for quite some time that beta cells have an inherently high level of proton leak (Affourtit and Brand 2008), it was unclear whether this leak plays a role in insulin secretion. Islets isolated from HFD mice have characteristically increased basal insulin secretion(Fex et al., 2007). Our data indicate that PTP mediated leak plays an important role in amplifying signals for insulin secretion under basal glucose, while perhaps serving as a detoxification system to protect beta cells from nutrient overload. Mitochondrial leak may result from various proteins, such as ion channels, and exchangers that may dissipate the membrane potential independently from ATP production. A

short list of putative candidates for mitochondrial proton leak includes UCP2, the ANT, NNT, and low conductance state PTP activity. In Chapter 4 we identify the PTP as a source of leak that contributes to signals for insulin secretion under low glucose. In the case of UCP2, it has already been reported in the literature that UCP2 beta cell specific KO islets do not have significant difference in leak as compared to their respective WT control islets under either basal or stimulatory levels of glucose (Robson-Doucette et al., 2011), ruling out the possibility that UCP2 contributes to proton leak.

A limitation of our study is that we have not explored the contributions to leak from other candidates such as the ANT, which is responsible for ~50-67% of leak in the basal state of muscle mitochondria (M. D. Brand et al., 2005). ANT has a role in insulin secretion that is mediated by PEP, since PEP is inhibitory for ADP/ATP exchange (Ewart, Yousufzai, Bradford, & Shrago, 1983). Given that mitochondrial PEP export may be mediated by either the ANT or CIC (Passarella, Atlante, Valenti, & de Bari, 2003), future work determining the contribution of ANT to leak would be interesting to explore via bongrekic acid, an established ANT inhibitor (Klingenberg & Buchholz, 1973).

The NNT, which consumes the membrane potential by generation of NADPH is likely another mechanism by which leak may occur. This is a limitation in our study, since our findings are obtained from islets isolated from the Jax labs

C57BL6J, which are NNT-deficient. A recent paper from Darrell Neufer's group has compared the responses of two mouse strains of C57BL6J and C57BL6NJ which are NNT deficient or not, respectively (Fisher-Wellman et al., 2016). The NNT-deficient strain was hypothesized to display increased susceptibility to HFD, since the NNT is known to detoxify peroxides (Ronchi et al., 2013). However, no difference in susceptibility to HFD metabolic stress was observed. Moreover, the NNT-deficient strain displayed compensatory redox buffering capacity attributed to increased SOD2 and IDH2 (Fisher-Wellman et al., 2016). It would be interesting and valuable to test the contribution of PTP mediated leak in islets that express NNT as compared to our studies, since NNT is expressed in human islets.

Role of Cyclophilin D in Metabolic Diseases

Chapter 4 of this thesis reveals that diet induced obesity increases islet expression of CypD, and increases islet sensitivity to PTP mediated leak. Furthermore, islets from HFD mice have increased basal insulin secretion(Fex et al., 2007). Moreover, ablation of CypD reduces insulin secretion and mitochondrial leak under basal glucose without altering either at stimulatory glucose levels. These findings uncover a novel target to influence basal hypersecretion of insulin in the pre-diabetic state. Identification of this target (PTP) enables an alternative hypothesis in which prevention of hyperinsulinemia

may delay the associated changes in insulin resistance. A whole body KO of CypD is protective from diet-induced obesity, attributed to increased skeletal muscle glucose uptake (Taddeo et al., 2014). It is unclear if this is due primarily to improvement in insulin sensitivity or reduction in mitochondrial dysfunction. The possibility exists that a reduction in beta cell basal insulin secretion may play a role. Moreover, ablation of CypD in a PDX-1 deletion diabetes model prevents beta cell death, restores beta cell mass, and normalizes fasting blood glucose (Fujimoto, Chen, Polonsky, & Dorn, 2010). CypD KO or pharmacological inhibition of the PTP is also protective from hepatic steatosis (X. Wang et al., 2018), further implicating the role of CypD in metabolic diseases. Future studies are needed to determine the contribution of CypD ablation on beta cell function and insulin sensitivity. It is noteworthy to mention that a study investigating the role for CypD on behavioral and neurological function uncovered a role for CypD in metabolism and energy expenditure upon observation of adult-onset obesity in KO mice. This was attributed to increased WAT and decreased locomotor activity (Luvisetto, Basso, Petronilli, Bernardi, & Forte, 2008). Understanding the discrepancies in these studies may lie in differences of the diet, age or gut micro biome and requires further work.

Pharmacological Implications of PTP Inhibition

In Chapter 4 we also reveal the possibility that inhibition of the PTP may be a therapeutic target to prevent fasting hyperinsulinemia during pre-diabetes. Our studies employed the use of NIM-811, a non-immunosuppressive compound that is a cyclosporine derivative (Waldmeier et al., 2002). Cyclosporine A has been widely utilized as an immunosuppressive agent for patients that undergo transplantation for many years. Studies conducted in the 80's discovered that CsA acutely inhibits GSIS in a dose dependent manner that is not reversible after washout in HIT cells and rat islets (Robertson, 1986). Chronic CsA treatment impairs islet proinsulin biosynthesis, and reduces GSIS and DNA synthesis(Andersson et al., 1984). While CsA displays negative effects on GSIS, we find that acute treatment of islets with its analog, NIM-811 reduces basal hypersecretion of insulin under stimulation with fatty acids or amino acids. The negative effects of CsA may be due to its inhibition of calcineurin, which does not occur with NIM-811. Inhibition of calcineurin prevents dephosphorylation of the mitochondrial fission protein dynamin related protein 1 (Drp1) at Serine 637, a requirement for Drp1 translocation to mitochondria to induce mitochondrial fragmentation (Cereghetti et al., 2008). Prevention of mitochondrial fragmentation will result in inhibition of autophagy (Twig & Shirihai, 2011), which is critical for

mitochondrial quality control and beta cell function under pathophysiological conditions(Las et al., 2011). Nevertheless, it remains to be determined if inhibition of CypD with NIM-811 will have similar effects in islets exposed to chronic nutrient excess.

Considerable attention has been dedicated to uncover various compounds for PTP inhibition, since protective effects are observed in many diseases and it has promising therapeutic potential (Šileikytė & Forte, 2016). NIM-811 displays anti-viral activities making it a therapeutic target for hepatitis B(Bouchard, Puro, Wang, & Schneider, 2003), hepatitis C (Ma et al., 2006), and HIV (Billich et al., 1995; Rosenwirth et al., 1994). NIM-811 may also exert anti-inflammatory protective effects in a CypD independent manner, as recently shown in a model of encephalomyelitis (Huang, Pandya, Banta, Ansari, & Oh, 2017). Acute administration of NIM811 has also shown protective effects in animal models of traumatic brain injury (Readnower et al., 2011). Furthermore, NIM-811 and its analog Debio 025, have shown promising protective effects on mitochondrial function in multiple models of muscular dystrophy (Tiepolo et al., 2009) (Zulian et al., 2014).

However, there are several challenges to the use of PTP inhibitors that target CypD (Šileikytė & Forte, 2016): 1) The essential structural components of the PTP and mechanism of opening have still not been resolved. 2) CypD is not

a core component of the pore, but a regulator; ablation of which serves to raise the calcium threshold at which PTP opening will occur. 3) Inhibitors of CypD are not only specific to CypD, but target all cyclophilins, making it difficult to ascertain whether the effects observed are due to PTP inhibition or involve other off target effects. 4) Other physiological functions of CypD have yet to be fully discovered (Javadov, Jang, Parodi-Rullán, Khuchua, & Kuznetsov, 2017). CypD does not serve solely as a regulator of the PTP, but also plays an important role in protein folding since it serves as a chaperone catalyzing isomerization of cis-trans peptidyl-prolyl bonds (P. Wang & Heitman, 2005). More recently, CypD has been shown to interact with mitochondrial transcription factors and influence expression of electron transport chain components such as NADH Dehydrogenase (ND1), cytochrome oxidase 1(COX1), and ATP synthase subunit 6 (ATP6) (Radhakrishnan, Bazarek, Chandran, & Gazmuri, 2015). Successful development of isoform specific inhibitors of cyclophilins (Daum et al., 2009), would be extremely valuable to prevent undesirable effects on functions of other cyclophilins which may be unfavorable. Future research employing the use of other CypD inhibitors, or inhibitors of the PTP which act independently of targeting CypD may be valuable to discern the differences. This may be challenging, however, since most high throughput screens for PTP inhibitors are conducted on isolated mitochondria, which require further validation on intact cells where permeability may be a concern (Šileikytė & Forte, 2016). Finally, the

use of these compounds may have entirely different effects on an organismal level, which needs to be tested.

Conclusions and Future Directions

The overarching objectives of this thesis were to probe mechanisms contributing to HI of the pre-diabetic state. Since there is no consensus on the primary defect (HI or IR) that drives progression of T2D, elucidating molecular mechanisms that govern insulin secretion in the basal state independently of GSIS would enable manipulation of these pathways to characterize their contributions. Current T2D therapies do not prevent progression of disease, but rather exhaust beta cell function ultimately resulting in beta cell failure, compelling patients to resort to exogenous insulin therapy. Careful consideration for models of beta cell glucolipotoxicity is necessary to achieve results that can be more physiologically extrapolated. Our discovery of the PTP as a novel regulator of basal insulin secretion enables the possibility to test hyperinsulinemia as a driver of the pre-diabetic state. Future studies further characterizing the role of the PTP in beta cell dysfunction has therapeutic potential, particularly since this mechanism is shown to be fundamental contributor to many diseases.

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